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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Urinary tract infections (UTIs), generally caused by Escherichia coli or Staphylococcus saprophyticus, are extremely common among young women. Although UTIs can be treated, we currently lack effective means to prevent frequently UTIs, which occur in 25% of women with first UTI. A necessary prerequisite to UTI is adherence of uropathogens to the vaginal and bladder epithelium. Preliminary studies show that glycosphingolipids (GSLs) are key host cell receptors for E. coli and S. saprophyticus. This final report describes progress in a project whose overall goal is to (1) show that globoseries and ganglioseries GSLs are present in primary cultures of human vaginal and bladder epithelial cells and characterize the GSLs; (2) define the roles of these GSLs in E. coli and S. saprophyticus UTI, using these culture systems; and (4) use these results to design new agents to prevent colonization and infection in women. We have developed these cultured cell systems as new models for studying UTI in women and have characterized GSL binding receptors in these cell cultures. We defined new functional aspects of these epithelia in UTI and developed methods for preparing blocking agents. These findings will be useful in further studies of UTI and other urogenital infections.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
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4. TABLE OF CONTENTS

5. INTRODUCTION

Although acute and recurrent UTIs are common, the only currently available preventive modality for recurrent UTIs is prophylaxis. An increasingly important concern about antimicrobial prophylaxis is its potential for promoting the emergence of drug-resistant strains (14). The studies described in this final report focused on investigating key aspects of the initial step in the pathogenesis of UTI as a point at which developing non-antibiotic preventive mechanisms appears feasible. This key step in uropathogenesis is the initial interaction of the infecting bacteria with urogenital epithelial cells of the host, an interaction that depends upon bacteria binding to host cell cognate receptors. It is a critical point in the process leading to UTI and also influences host susceptibility to infection. Among key host cell cognate receptors for bacteria causing UTIs are glycosphingolipids (GSLs), components of the mammalian glycocalyx consisting of a lipid embedded in the plasma membrane linked to an oligosaccharide moiety exposed on the cell surface. GSLs serve as eukaryotic cell adhesion sites for many pathogens and are tissue-specific and/or genetically determined (5). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases such as UTI.

GSLs are grouped into families based on their core carbohydrate structures. We and other investigators showed that GSLs of the globoseries family serve as receptors for uropathogenic E. coli (6-8, 16, 18), and our preliminary data demonstrated that Staphylococcus saprophyticus binds to ganglioseries GSLs. In the bladder, GSLs of other families (lactoseries and neolactoseries) have been investigated because these GSLs are altered during oncogenesis. Suitable materials are available for the study of these GSLs, such as continuous cell lines and tumor samples. However, comparable tools have not been available for the study of normal bladder or vaginal epithelial cells. In addition, the globoseries and ganglioseries families of GSLs have not been studied in these tissues. The studies outlined in this report were designed to address these deficiencies by synthesizing our ongoing collaborations and ideas from the diverse fields of glycobiology, chemistry, urology, and gynecology to carry out this project. Contemporaneously with the first four years of funding, Dr. David Eschenbach of the Department of OB-GYN at the University of Washington investigated key aspects the native vaginal epithelium, in collaboration with Dr. Stapleton and others (2-4, 10). These were the first systematic studies of the vagina in many years. The findings of these studies were invaluable to us in developing conditions for maintaining vaginal epithelial cells in vitro, in itself a new technique developed through the studies described in this report. In addition, we are the only group to characterize the expression of GSLs in native vaginal epithelial cells (16) and our collaboration with Dr. Atala to use primary cultured bladder epithelial cells as a system to characterize bladder GSLs is also unique.

We combined these unique resources to accomplish the following goals: (1) to investigate the interactions between the two bacteria causing most urinary tract infections (UTIs) in young women, Escherichia coli and Staphylococcus saprophyticus, and their cognate glycosphingolipid (GSL) host cell receptors in the vaginal and bladder epithelium (2) to use this information to design novel, non-antibiotic methods for preventing UTIs. The hypotheses originally proposed are as follows: (1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for E. coli and S. saprophyticus, respectively; (2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection; (3) We hypothesize that carbohydrate mimetic and synthesis

techniques can be used to design high-affinity inhibitors of E. coli and S. saprophyticus binding to vaginal and bladder transitional epithelium.

Please note that although all prior reports have been approved, the format of this report has been changed in response to the most recent review. This reviewer made specific requests for formatting, such as detailing methods in references. In addition, a comment was made on the exhaustive nature of the prior report. These recommendations have been incorporated into the formatting of this final report. All sections have been written as concisely as possible. Wherever appropriate, a summary approach has been taken, referring to prior reports and/or publications for details, as outlined in the instructions for final reports (part b).

6. BODY OF REPORT

A. Overview

Review of changes and opportunities occurring throughout the project funding:

1. New collaborations and new approaches: we established several new collaborations during the course of the project to assist us in performing the proposed studies. These collaborations assisted in overcoming key technical hurdles and also resulted in manuscripts being published or in preparation. In some cases, we used different technical approached in accomplishing some of the tasks, providing us with new opportunities. New collaborators and opportunities we utilized are as follows:

- GSL chemistry work: Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens and members of the Mass Spectrometry Laboratory in the Department of Medicinal Chemistry at the University of Washington have assisted us with structural characterization of GSL and with developing matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technology for new applications characterizing GSLs in complex mixtures.

- Vaginal epithelial cell cultures: Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchison Cancer Research Institute and the University of Washington and several members of Department of Obstetrics and Gynecology at the University of Washington assisted us with instituting and developing primary cultures of vaginal epithelial cells, primarily by providing discarded tissues needed for the establishment of these cell cultures. We have recently completed the characterization of keratin expression in cultured vaginal epithelial cells with the assistance of Dr Beverly Dale-Crunk, Professor of Oral Biology and Scientific Director of the Comprehensive Oral Research Center at the University of Washington.

- Bladder epithelial cell cultures: We continued work with Dr. Atala, as originally proposed, and have also established a local collaboration with Dr. Richard Grady, Assistant Professor of Urology at the University of Washington and Children's Hospital and Regional Medical Center. With Dr. Grady's assistance, we were able to institute the establishment of cultured bladder epithelial cells in our own institution, rather than using only cells originally established by our collaborator and shipped to us.

2. New approaches taken by Dr. Stroud: because of a combination of technical challenges and developments in the field, Dr. Stroud has further altered his approaches to Technical Objective 3, detailed below.

3. Personnel issues: the environment in which Dr. Stroud works has been less than optimal in the past two years because the group first moved its laboratory facilities twice in one year, then experienced the death of their chairman. We ameliorated these difficulties as best we could, in part with the help of our extensive collaborations, and Dr. Stroud was able to develop innovative approaches to the tasks he was assigned, summarized below.

B. Progress on Specific Tasks

1. Technical Objective 1

a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala during the first two years of funding and now have been established as a routine technique in our own facility. Dr. Grady has assisted us in acquiring discarded tissue samples since 1999, under an IRB protocol for which he is principal investigator in his institution. We have adopted Dr. Atala's methods and are now independently establishing and maintained cultures in serum free keratinocyte media using standard tissue culture technique, as originally described (1), with occasional collaborative advice from Dr. Atala.

2. Results and discussion

Bladder epithelial cells: Data from the studies utilizing these cells are incorporated into a manuscript in preparation.

Vaginal epithelial cells: We have fully established a culture method using surgical specimens from women without cervical or vaginal malignancy, infection, or other non-anatomic disorders of the vagina, obtained in accordance with human subjects regulations in our institution. Cell cultures can be maintained in serum free keratinocyte medium up to seven passages. In the first four progress reports, we described various technical difficulties, means of overcoming problems, and progress in culturing vaginal epithelial cells, e.g. methods for cell seeding, avoidance of contamination, and eliminating fibroblast contamination. All difficulties have been overcome. Frozen stocks of primary vaginal epithelial cells have been obtained by cryopreservation of early passage cells, using methods we routinely perform for bladder epithelial cells. Twenty-four cultures are presently stocked. A description of the methods will be submitted for publication in the near future, incorporated with data describing the characterization of the differentiation state of these cells.

Further studies: We have pursued a more formal investigation of the state of differentiation of the cultured vaginal epithelial cells after discussions with various colleagues, especially Dr. T.T. Sun of New York University and Dr. Beverly Dale, Professor of Oral Biology at the University of Washington, both experts in epithelial biology. Although our tissue culture techniques are standard, methods for culturing

primary vaginal epithelial cells have not been previously reported. In addition, little baseline information is available regarding keratin expression and other possible markers of differentiation in the vaginal epithelium. Our colleagues felt that before publication of any data generated in this system, it would be critical to characterize these cells and to investigate native vaginal tissue itself using methods applied to other epithelial culture systems, such as keratinocytes and oral epithelial cells. Thus, Dr. Dale graciously agreed to collaborate to assist in the somewhat laborious task of characterizing our cultured vaginal epithelial cell system. Thus, we have characterized cytokeratin expression in the cultured vaginal epithelial cell system. Some of these data were described in detail in the annual reports for 1999 and 2000. A brief summary follows.

During our initial efforts to successfully isolate and maintain vaginal epithelial cells, prior to our collaboration with Dr. Dale, we used a Mab that stains all epithelia (PAN; Sigma) to stain our cell cultures, in parallel with pure cultures of human fibroblasts. These studies showed that the vaginal epithelial cells stained positive with this Mab but not with an antibody directed against fibroblasts, while fibroblasts in pure culture did not stain with PAN but did stain positive with the anti-fibroblast MAb. Thus, we showed that the primary cultured vaginal epithelial cells were epithelial cells and not fibroblasts (data shown in the progress report for Year 2). Subsequently, to fully characterized the cells' keratin expression, we grew two separate cell cultures under various conditions designed to alter the degree of differentiation, such as with and without the addition of 17-ß-estradiol in physiological concentrations, or fetal calf serum in concentrations that cause differentiation of other stratified epithelia in culture. We then extracted these cell cultures for keratins and performed a series of standard Western blots using Mabs directed against K1, K5/8, K6, K8 alone, K10, K13, K14, K16, K18, and K19. The methods for these procedures and some of the results were described in the report for year 4. These Mabs were chosen in consultation with Dr. Dale, based on the small amount of information available about the vaginal epithelium combined with data on keratins as differentiation markers in other epithelia. In the past year of funding, we have completed these studies and performed parallel studies in which we stained cultured cells and tissue sections from normal women with key antibodies identified from the Western blot studies. As noted above, surprisingly few previous studies have been published characterizing any keratins in the vaginal epithelium. Thus, our work is a major contribution to the field of women's health.

We are in the process of assembling a manuscript based on these data, which will credit this funding source when it is submitted in the next several months. The data are complex and have been partially presented in prior reports. In summary, our work demonstrates that vaginal epithelial cells grown in culture without added serum or estrogen maintain an intermediate level of differentiation. Cells then become terminally differentiated to a suprabasal and superficial phenotype under the influence of serum or estrogen. This is illustrated by results of staining results with Mabs directed against K19, (expected to be found expressed in basal cells K13), K14 (found in cells of varying degrees of differentiation) and K13 (expected to be expressed in suprabasal cells). The cells express K19 when grown in conditions producing a basal, undifferentiated morphology but do not express K13 or K14. These patterns reverse when the cells are grown in serum or 17-ß-estradiol.

Additional data below describing bacterial adherence and GSL characterization studies complete the establishment of our method of culturing vaginal epithelial cells as a model for the study of colonization and adherence in UTI and other urogenital infections.

b. Task 2, Months 7 to 12: extraction and purification of GSLs from bladder and vaginal cell cultures;
c. Task 3, Months 7 to 12: bacterial overlay assays;
d. Task 4, Months 7 to 12: immunostaining assays;
e. Task 5, Months 13 to 24: carbohydrate structural analysis; and
f. Task 6, Months 25 to 36: data analysis and publication

1. Experimental methods, assumptions and procedure

Methods for the extraction and purification of GSLs from cells, as well as bacterial overlay and immunostaining assays are as described in two prior publications (15, 17) which are appended.

2. Results and discussion

The planned studies for tasks **b** to **e** utilizing bladder epithelial cells have been completed, as have bacterial overlay assays with E. coli and S. saprophyticus, and tasks outlined below in Technical Objective 2. We are preparing two separate manuscripts describing these data, in combination with results from Technical Objective 2. Most of the results have been described in prior reports, including figures. Summaries of the two manuscripts are provided below, and a figure showing some newly obtained GSL structural data is provided. Data related to Technical Objective 2 are incorporated into Manuscript 1, but are described in the section for Technical Objective 2, for convenience. Studies related to vaginal epithelial cells have been incorporated into a draft of a third manuscript, also summarized below.

Manuscript 1. P fimbriated Escherichia coli bind to globoseries glycosphingolipids in cultured primary bladder epithelial cells: a new model for bladder infection (Stapleton et al.)

a. GSLs in primary cultured bladder epithelial cells differ from those in bladder cell lines T24 and J82. To demonstrate the utility and relevance of our primary cultured bladder epithelial cells, we compared GSL profiles from cultured bladder epithelial cells with GSLs extracted from continuous bladder epithelial cell lines T24 and J82, available from ATCC and previously used in in vitro models of UTI. From these experiments, we concluded that cultured primary bladder epithelial cells contain globoseries GSLs that bind P fimbriated uropathogenic E. coli. In addition, primary cultured bladder epithelial cells are enriched in extended globoseries GSLs as compared with T24 and J82 cells. The GSLs identified in normal cells including SGG, previously shown to be high avidity GSL ligand for uropathogenic E. coli in native vaginal epithelial cells (16, 17).

b. Binding of E. coli R45 to GSLs extracted from multiple individuals confirms the expression of globoseries GSLs and identifies these moieties based on bacterial binding, Mab staining, and mobilities on HPTLC. Bacterial overlay assays performed on a total of 14 primary bladder epithelial cell cultures established from separate individuals confirmed that primary cultured bladder epithelial cells contain appropriate globoseries GSLs, based on binding of bacteria and co-mobilities, and immunostaining assays with Mabs ID4 (directed against SGG) and MC631 (directed against related extended globoseries GSLs (9)). No individual variation was seen in expression of GSLs whose expression is predicted to be conserved regardless of secretor status or ABO type, confirming the stability and reliability of this system.

Note: remaining portions of this draft manuscript (data on bacterial adherence assays and carbohydrate structural analysis) are outlined below in the relevant sections of this report.

Manuscript 2. Role of asialo GM1 binding in the pathogenesis of Staphylococcus saprophyticus urinary tract infection. (Stapleton et al.)

This manuscript described bacterial overlay and adherence assays with S. saprophyticus, utilizing primary cultured bladder epithelial cells. We studied S. saprophyticus ST352, a representative cystitis isolate that we found to be highly adherent to native vaginal epithelial cells. Testing the binding of this organism to standard GSL samples extracted and purified from various sources, we found that S. saprophyticus ST352 binds the ganglioseries GSL asialo GM1 (ASGM1). We also tested three other S. saprophyticus cystitis isolates that were also highly adherent to vaginal epithelial cells. These isolates also bound ASGM1, and two organisms also bound other structurally related ganglioseries GSLs. We used cholera toxin binding and immunostaining to find that GM1 and ASGM1, respectively, are present in GSLs extracted from vaginal epithelial cells and from human kidney tissues. ASGM1 was surface exposed and co-localized with areas of S. saprophyticus adherence in human kidney sections. These data demonstrate that ASGM1 and related ganglioseries structures serve as receptor(s) for S. saprophyticus binding, at least in the kidney.

Manuscript 3. Bacterial adherence in a new model of primary vaginal epithelial cells

The establishment of our system for the primary culture of vaginal epithelial cells and the characterization of the cells were described above. We have also shown that uropathogenic E. coli adhere in higher numbers to vaginal epithelial cells grown without 17-ß-estradiol. These cells showed a basal morphology and expressed keratins more commonly found in basal cells of related stratified epithelia, as noted above. Conversely, when vaginal epithelial cells were maintained for 3 days in physiological concentrations of 17-ß-estradiol, adherence of uropathogenic E. coli was reduced. Concomitant analysis of GSL expression in these cells showed that estrogen exposure reduced overall expression of GSLs and altered the globoseries expression pattern towards shorter chain, lower affinity GSLs. Thus, primary vaginal epithelial cells grown in culture and exposed to estrogen were relatively resistant to bacterial attachment, and this finding correlated with changes in GSL receptor repertoire for the attaching bacteria. These findings correlate with unpublished clinical data from our group, showing that native exfoliated vaginal epithelial cells collected from post-menopausal women not taking hormone replacement therapy (unestrogenized) are more receptive to adherence by uropathogenic E. coli than are cells collected from pre-menopausal, estrogenized women. This in vitro effect of estrogen may explain why the risk of UTI increases post-menopause, but can be ameliorated by topical vaginal estrogen administration (11,12).

e. Task 5, Months 13 to 24: carbohydrate structural analysis (additional details)

1. Experimental methods, assumptions and procedure; and
2. Results and discussion

Methods for structural analysis of carbohydrates are referenced in the appended manuscripts. In prior reports, we described methods for sequencing carbohydrate portions of GSLs using serial exoglycosidase experiments. We pursued this method sufficiently to determine that it was excessively costly of

enzymes and of the GSL substrate we painstakingly prepared. Dr. Stroud subsequently developed other methods of producing larger amounts of substrate that proved useful in the approach we ultimately adopted, that of using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry performed on complex mixtures of GSLs. With the assistance of colleagues in the Mass Spectrometry Laboratory in the Department of Medicinal Chemistry, Dr. Stroud applied a delayed ion extraction (DE) technique that dramatically improved the resolution and accuracy of mass spectra we obtained. This technique allowed us to analyze the individual molecular species of a variety of native GSLs within a mixture. Various purified neutral and monosialylated GSLs were examined by MALDI-TOF in the reflector mode. Native globoseries GSLs, Gb_4 and Gb_5, and the monosialyl ganglioside GM_1 gave good spectra in the positive ion mode (detection limits of 10-100 pmol). Analysis of a total GSL fraction isolated from a human primary bladder cell line also gave a good spectrum of GSL derived molecular ions, as shown below in Figure 1. These data are also incorporated into our Manuscript 1 described above.

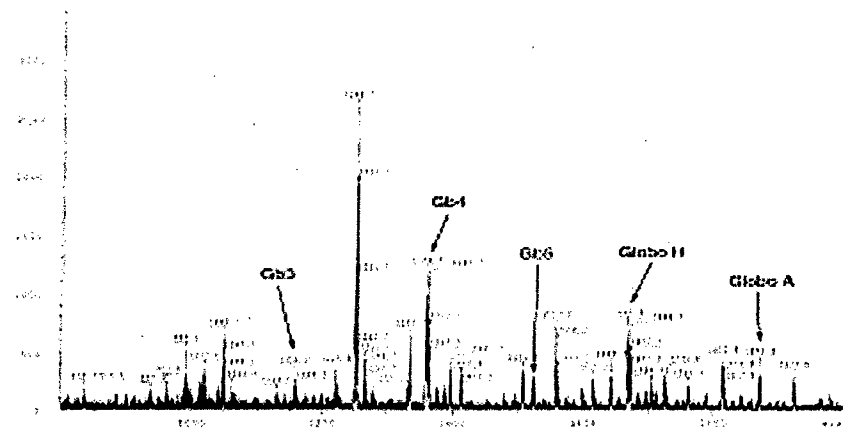

Figure 1. MALDI-TOF analysis of GSLS extracted from cultured primary bladder epithelial cells. One microliter of a mixture of glycosphingolipids isolated from a human primary bladder epithelial cell culture dissolved in chloroform:methanol (2:1) was mixed with 1 μL of matrix solution (10 mg/mL) of 2,5-dihydroxybenzoic acid. One μL of the glycolipid/matrix solution was added onto a single well of a sample plate and allowed to dry and crystallize at room temperature. The sample plate was loaded into a Bruker Biflex-III MALDI-TOF mass spectrometer and a 337 nm nitrogen laser was used for ionization with a 270 cm effective flight path in the reflectron mode. Data was collected over 500 hits by the laser in the positive-ion mode. Laser attenuation was adjusted to 80 units at the start of acquisition and decreased accordingly by 5 units until the spectrum was optimized. A two point external calibration was performed each time.

2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures;
b. Task 2, Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs;
c. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays

1. Experimental methods, assumptions and procedure; and
2. Results and discussion; and

Methods are as described above and in the appended papers. Some data describing bacterial adherence experiments and immunostaining of cells were presented in prior reports. Additional relevant results were incorporated into the above-described manuscript, summarized as follows:

Manuscript 3, part c. The adherence of P fimbriated E. coli to primary bladder epithelial cells depends on the expression of globoseries GSLs. We cultured primary bladder epithelial cells with and without PDMP, an inhibitor of GSL synthesis. Uropathogenic P fimbriated E. coli adhered reproducibly to the cells in high numbers in the absence of PDMP, but adherence was essentially absent in the presence of PDMP (data presented in prior reports). Parallel GSL extraction studies from cells grown with and without PDMP showed that the synthesis of globoseries GSLs was dramatically reduced during growth in PDMP. These findings demonstrate the dependence of uropathogenic E. coli adherence on the expression of globoseries GSLs in primary bladder epithelial cells.

Note that similar experiments were performed using primary cultured vaginal epithelial cells grown with and without PPPP, a related compound, producing similar results.

3. Technical Objective 3

a. Task 1, Months 24 to 42: synthesis of linear mimetic compounds

1. Experimental methods, assumptions and procedure; and
2. Results and discussion

Dr. Stroud has had difficulty in pursuing synthesis aspects of this task according to the original methods we proposed. Thus, he has sought assistance from various colleagues, especially Dr. Henrik Clausen at the University of Copenhagen, with whom he recently published data relevant to these studies In a prior report, we described methods Dr. Stroud developed for the enzymatic synthesis of globoseries based GSL receptor analogs. Dr. Stroud has succeeded in producing milligram quantities of Gb_4 and Gb_5 using this technique, but we have prioritized the use of these compounds to the MALDI studies described above. Given that this enzymatic method has limitations in the time and labor it requires, Dr. Stroud has pursued an alternate strategy in the past few months. A brief outline of his methods are as follows: Dr. Stroud has worked out conditions to effectively release the free oligosaccharide from the ceramide moiety of Gb_4 using ceramide glycanase from leaches. He has prepared approximately 2 mg of free Gb_4 oligosaccharide using this method. The oligosaccharide will be covalently coupled to lysyllisine by reductive amination as described previously by Schwartz and Gray (13). This method is based on the ability of cyanoborohydride to reduce a Schiff base selectively at $pH > 5$. Briefly, unreduced oligosaccharide, L-lysyllisine, and sodiumborohydride will be mixed in a molar ratio of 0.35:0.033:1.00 and dissolved in 0.2 M potassium phosphate. After 72 h at 37 C, the oligosaccharide-lysyllysine conjugate will be purified by P2 column chromatography. We will then be able to us this multivalent conjugate in E. coli binding inhibition studies, using either or both cell cultures systems. Various Gb_4 derivatives will also be used with this method. We hope to complete these studies in the next 3 to 4 months.

7. KEY RESEARCH ACCOMPLISHMENTS

1. Establishment of in vitro models of human primary cultured bladder and vaginal epithelial cells

- Characterization of glycosphingolipids (GSLs) expressed by these epithelial cells
- Characterization of bacterial adherence to primary cultured bladder and vaginal epithelial cells by two organisms that are key uropathogens for healthy young women, E. coli and S. saprophyticus

2. Establishment of the principle that GSLs are essential for the adherence of P fimbriated uropathogenic E. coli in primary bladder and vaginal epithelial cells

3. Studies of the effects of exogenous estrogen on the expression of GSLs and keratins in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on bacterial adherence

4. Cloning of a human α1-4Galactosyltransferase relevant to synthesis of globoseries based GSL receptors

- Enzymatic synthesis of globoseries based GSL receptors and development of multivalent conjugate forms of oligosaccharide receptor analogues for use in inhibition studies
- Development of MALDI mass spectrometry to characterize complex mixtures of native GSLs

8. REPORTABLE OUTCOMES

a. Manuscripts, abstracts and presentations

Manuscripts

1. Hooton TM, Scholes D, Hughes JP, Winter C, Roberts P, Stapleton A, Stergachis A, Stamm WE. A prospective study of risk factors for urinary tract infection in young women. N Engl J Med 1996; 335:468-474.
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Presentations and Meetings

- Moderator and Scientific Committee, 2000 and 2001 International Bladder Symposia
- Invited Speaker, Second International Bladder Research Congress, San Francisco, April 1998.
- Invited Speaker, Rodin Conference, July 1997 and July 2000
- Invited speaker, The Millennium Symposium of Pyelonephritis and UTI, Lund, Sweden, May 2000
- Invited speaker, University of Washington Science in Medicine New Investigator series, 1999-2000 (also received award in connection with this lecture series)

- Obstetrics and Gynecology Grand Rounds, 2000
- Poster presentation, 2001 International Bladder Symposium

Development of cell lines

Primary cultures of vaginal epithelial cells derived from individuals have been developed for the purposes of these studies. However, these cells have not been provided to other investigators as a "reagent" and are not suitable for an application of that nature, such as submission to a cell banking facility.

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Ann Stapleton, MD
Mark Stroud, PhD
Amy Denton, BS
Michelle Brown, BS
Afshin Mahmoudi, MS

9. CONCLUSIONS and "so what" comments

Our initial years of funding were focused on refining the techniques we developed for culturing primary vaginal epithelial cells and on adapting Dr. Atala's methods for culturing primary human urothelial cells to our model system. We have now established these in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells for the identification, purification and structural characterization of E. coli- and S. saprophyticus-binding glycosphingolipid moieties. These models will be valuable for future research in UTI and other urogenital infections and will also contribute to basic knowledge about the bladder and the vagina. We have completed studies characterizing bacterial adherence to primary bladder and vaginal epithelial cells utilizing organisms that are key uropathogens for healthy young women, E. coli and S. saprophyticus. Findings in these systems will also be applicable to the study of other mucosal infections with E. coli and other gram negative pathogens, and may also be applicable to studies of Staphylococcus aureus. We have also established that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems. We have completed characterization of primary cultured vaginal epithelial cells according to parameters normally applied to similar epithelia, in collaboration with respected investigators in the field of epithelial biology. We have also characterized the effects of exogenous estrogen on the expression of GSLs and keratins as well as on bacterial adherence in vaginal epithelial cells, all of which are relevant to the use of these cells as models of UTI and other urogenital diseases throughout the life cycle. This model of the vaginal epithelium provides new opportunities for studying other aspects of women's urogenital health, including testing vaginal products, probiotics, and contraceptives; understanding protective roles of organisms in the normal flora; and studying the cellular effects of hormone replacement therapy on a key target tissue, the vagina. Lastly, we have advanced the study of the globoseries GSLs, which have not been thoroughly studied in normal adult tissues. Thus, our findings have additional relevance in the field of glycobiology.

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11. APPENDICES

Appendix A: reprints of the selected publications listed below are appended to facilitate the description of methods. References 1,2 and 3 describe methods for the extraction and purification of GSLs, bacterial overlay and immunostaining procedures, and immunocytochemical staining methods. Reference 4 provides an overview and perspective on the prevention of UTI. Reference 5 provides background information on uropathogenic E. coli directly relevant to this project.

1. Stapleton AE, Stroud MR, Hakomori SI, Stamm WE. The globo-series glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor in vitro for uropathogenic Escherichia coli expressing pap-encoded adhesins. Infect Immun 1998;66:3856-3861.

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The Globoseries Glycosphingolipid Sialosyl Galactosyl Globoside Is Found in Urinary Tract Tissues and Is a Preferred Binding Receptor In Vitro for Uropathogenic *Escherichia coli* Expressing *pap*-Encoded Adhesins

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Women with a history of recurrent *Escherichia coli* urinary tract infections (UTIs) are significantly more likely to be nonsecretors of blood group antigens than are women without such a history, and vaginal epithelial cells (VEC) from women who are nonsecretors show enhanced adherence of uropathogenic *E. coli* isolates compared with cells from secretors. We previously extracted glycosphingolipids (GSLs) from native VEC and determined that nonsecretors (but not secretors) selectively express two extended globoseries GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG), which specifically bound uropathogenic *E. coli* R45 expressing a P adhesin. In this study, we demonstrated, by purifying the compounds from this source, that SGG and DSGG are expressed in human kidney tissue. We also demonstrated that SGG and DSGG isolated from human kidneys bind uropathogenic *E. coli* isolates expressing each of the three classes of *pap*-encoded adhesins, including cloned isolates expressing PapG from J96, PrsG from J96, and PapG from IA2, and the wild-type isolates IA2 and R45. We metabolically ^{35}S labeled these five *E. coli* isolates and measured their relative binding affinities to serial dilutions of SGG and DSGG as well as to globotriaosylceramide (Gb_3) and globotetraosylceramide (Gb_4), two other globoseries GSLs present in urogenital tissues. Each of the five *E. coli* isolates bound to SGG with the highest apparent avidity compared with their binding to DSGG, Gb_3, and Gb_4, and each isolate had a unique pattern of GSL binding affinity. These studies further suggest that SGG likely plays an important role in the pathogenesis of UTI and that its presence may account for the increased binding of *E. coli* to uroepithelial cells from nonsecretors and for the increased susceptibility of nonsecretors to recurrent UTI.

Several epidemiological studies have shown that women who are nonsecretors of blood group antigens have a three- to fourfold-increased risk of developing recurrent urinary tract infection (UTI) (5, 17, 32). In addition, uroepithelial cells from nonsecretors have a two- to threefold-greater capacity for adherence of uropathogenic *Escherichia coli* than do cells from secretors (22). Colonization of the vaginal and periurethral epithelium precedes the development of *E. coli* UTI, and *E. coli* isolates expressing *pap*-encoded adhesins are overrepresented among isolates causing these infections (6). Uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins bind to globoseries glycosphingolipids (GSLs) (6, 19), which are amphipathic molecules embedded in the outer leaflets of eukaryotic cell membranes. There are several families of GSLs which are differentiated by their molecular structures, and these molecules serve as bacterial and viral adhesion sites on mammalian cells and as markers of eukaryotic cell differentiation and oncogenesis (4).

In previous investigations, we collected vaginal epithelial cells from secretors and nonsecretors and extracted the GSLs from pooled cells from women in each group (36). We demonstrated that cells from nonsecretors express two unique globoseries GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG) (36). We utilized high-performance thin-layer chromatography (HPTLC), bacterial overlay assays, HPTLC immunostaining, radioimmunoassays, and immunohistochemical staining with a monoclonal antibody (MAb) directed against SGG to show that SGG and DSGG were expressed in vaginal epithelial cells from nonsecretors but not in cells from secretors and that these moieties bound a clinical isolate of *E. coli* (R45) that expresses P fimbriae carrying a *pap*-encoded adhesin (36). These studies demonstrated for the first time that the secretor gene influences the biosynthesis of globoseries GSLs in the vaginal epithelium and suggested that genetically determined differences in receptor moieties in this tissue might explain the increased susceptibility of nonsecretors to UTI (32, 36).

In this study, we isolated and purified SGG and DSGG from human kidneys and assessed the in vitro binding of representative Pap-expressing *E. coli* isolates to SGG and DSGG in order to further elucidate possible mechanisms through which the selective expression of one or both of these molecules in the vaginal or urogenital epithelium of nonsecretors might influence their risk of UTI.

(This work was presented in part at the 32nd annual meeting of the Infectious Diseases Society of America [36a].)

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TABLE 1. Structures of GSL standards used in this study

GSL[a]	Structure[b]	Source
CMH	Glcβ1-1cer	Colonic adenocarcinoma
CTH	Galα1-4 Galβ1-4 Glcβ1-1cer	Human erythrocytes
Globoside	GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human erythrocytes
Gal globoside	Galβ1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-cer	Human kidney
SGG	NeuAcα2-3 Galβ1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human kidney
DSGG	NeuAcα2-3 Galβ1-3 (NeuAcα2-6) GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Human kidney
Forssman	GalNAcα1-3 GalNAcβ1-3 Galα1-4 Galβ1-4 Glcβ1-1cer	Goat erythrocytes
ASGM1	Galβ1-3 GalNAcβ1-4 Galβ1-4 Glcβ1-1cer	Desialylated GM1 from bovine brain

[a] Globoside, Gb_4 (globotetraosylceramide); Gal globoside, galactosyl globoside; ASGM1, asialo-GM1.
[b] Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic (sialic) acid; cer, ceramide.

MATERIALS AND METHODS

Purification of SGG and DSGG from human kidney tissue. Normal human kidney tissue was chosen as an appropriate source from which to purify SGG and DSGG for several reasons. First, it is an available and clinically relevant urinary tract tissue, whereas the vaginal epithelium cannot be harvested in sufficient quantity for the purification of SGG and DSGG. In addition, we chose a human tissue as the source for these compounds, since variations in the structure of the ceramide portions of GSLs may be species specific, and thus structural differences found in animal tissues can have implications for the binding specificities of microorganisms (14). In preliminary studies, using the methods described below, we extracted and purified GSLs from small autopsy samples of normal human kidney tissue and determined that SGG and DSGG were expressed in these tissues. The purification was then scaled up, and a total of 1 kg of normal human kidney tissue was obtained and pooled from autopsy specimens from eight individuals. Autopsy reports were reviewed to insure that none of the patients died from renal disease or from diseases affecting kidney function. The majority of the material by weight was obtained from a 38-year-old woman who died from medulloblastoma. The tissue was washed and homogenized in a Waring blender, and GSLs were then prepared by a series of standard purification steps. First, an organic extraction with isopropanol-hexane-water was performed (10), followed by a modified Folch extraction (3) to produce lower and upper phases. No further purification of the lower phase was performed for these studies. The upper phase was then subjected to anion-exchange chromatography (41). Neutral GSL fractions were collected in the flowthrough, and acidic fractions were eluted with 0.05, 0.15, and 0.45 M ammonium acetate washes. The neutral fraction was then further purified by reverse-phase chromatography followed by acetylation and deacetylation to remove phospholipids and cholesterol (40, 41). The acidic fractions were then subjected to normal-phase silica gel high-performance liquid chromatography (HPLC) (13). SGG and DSGG were then identified and purified from the HPLC fractions by stepwise combinations of HPTLC immunostaining (12, 24), bacterial overlay assays (36), HPTLC in multiple solvent systems, and preparative HPTLC (28). The purification of SGG and DSGG as well as the structural characterization of SGG will be described more fully elsewhere (37a).

HPTLC immunostaining and bacterial overlay assays. GSLs isolated and purified from the kidney tissues and then separated on HPTLC were immunostained according to the procedure of Magnani et al. (24), as modified by Kannagi et al. (12). Briefly, after HPTLC, the plates were blocked for 2 h in 5% bovine serum albumin in phosphate-buffered saline, washed, and incubated with the primary MAb in phosphate-buffered saline. After an incubation with the secondary antibody, the plates were washed, incubated with ^{125}I-labeled protein A solution, washed, dried, and subjected to autoradiography. MAbs ID4 and RM-1, directed against SGG (31, 36), were used to monitor the purification of both SGG and DSGG. Since DSGG differs in structure from SGG by only one sialic acid residue, DSGG was identified by subjecting the compound to a timed, limited desialylation procedure to produce SGG (27). Briefly, aliquots of the purified putative DSGG compound to be tested were incubated in 1% acetic acid for 1, 3, and 7 min at 100°C and the reactions were terminated by plunging the tubes in ice and adding ice-cold ethanol. The samples were then dried and subjected to HPTLC, and immunostaining with MAb ID4 was performed. The presence of globoseries GSL moieties, particularly SGG and DSGG, was also monitored in the various fractions obtained during the lengthy purification steps with HPTLC bacterial overlay assays. Assays were performed as previously described (36) with metabolically ^{35}S-labeled *E. coli* isolate R45, a wild-type cystitis isolate (35) which expresses P fimbriae carrying the class II *pap*-encoded adhesin (9) and binds globoseries GSLs (36). At every step, the results of HPTLC immunostaining and bacterial overlay experiments were compared, and relevant fractions and individual bands visualized by HPTLC were then subjected to further purification, as described above.

Bacterial binding curves. (i) GSL standards. Globoseries GSL standards were isolated and purified in our laboratory from the following sources, using methods similar to those described above for purifying SGG from human kidney tissue (29): (i) ceramide trihexoside (CTH; globotriaosylceramide [Gb_3]), from human erythrocytes; (ii) globoside (globotetraosylceramide [Gb_4]), from human erythrocytes; and (iii) SGG and DSGG, purified from human kidney tissue as described above. Ceramide monohexoside (CMH) was purified from colonic adenocarcinoma and was used as a negative control for the binding of *E. coli* expressing P fimbriae carrying *pap*-encoded adhesins (36, 37). GSL standards were quantitated by a combination of the resorcinol and sphingosine assays (25) and densitometry. Relative quantities of GSLs were standardized by HPTLC by the comparative dilution method, using an appropriate reference GSL having a carbohydrate chain of equal length and charge and of similar molarity to that of the GSL being standardized. (34). The structures of the compounds utilized are shown in Table 1.

(ii) Bacterial binding assays. To construct binding curves, GSL standards were serially diluted on HPTLC plates from 300 to 18.25 ng and overlaid with metabolically ^{35}S-labeled *E. coli* isolates in bacterial overlay assays, as previously described (36). This range of GSL concentrations was chosen on the basis of preliminary experiments with two of the *E. coli* isolates described below that showed saturation of bacterial binding for SGG at higher concentration ranges of these GSL standards. After bacterial overlay, the HPTLC plates were subjected to autoradiography, and densitometry of the autoradiographs was performed to assess the quantity of bacterial binding to each GSL relative to the others. A second method of assessing bacterial binding, using the same plates, was performed by scraping the silica band corresponding to bacterial binding to each GSL standard, followed by counting the radioactivity in a scintillation counter.

***E. coli* isolates.** The *E. coli* isolates that were tested included the following: (i) R45, a wild-type cystitis isolate from a woman with acute cystitis which expresses P fimbriae carrying a class II *pap*-encoded adhesin (8, 35); (ii) IA2, a second wild-type isolate, from which HB101/pDC1 (called pDC1 in this paper) was cloned and which expresses P fimbriae carrying a class II *pap*-encoded adhesin (2); (iii) JJ122, which expresses P fimbriae carrying a class I *papG*-encoded adhesin (PapG from J96) (HB101/pJJ48); (iv) pDC1, which expresses P fimbriae carrying a class II *pap*-encoded adhesin (PapG from IA2) (2); (v) P678-54/pJFK102 (called pJFK102 in this paper), which expresses P fimbriae carrying a class III adhesin (PrsG from J96) (15); and (vi) the negative control isolates HB101 and P678-54. Wild-type organisms were grown on sheep's blood agar, and recombinant isolates were grown on Luria broth agar plates containing the appropriate antibiotics. Under the growth conditions utilized for these studies, type 1 fimbriae were not expressed by any of the isolates (data not shown).

RESULTS

Purification of SGG and DSGG from human kidney tissue. As described above, the purification of SGG and DSGG was monitored by HPTLC immunostaining and bacterial overlay assays on fractions putatively containing the compounds of interest. The results of performing HPTLC immunostaining on samples of purified SGG and DSGG, using MAb RM-1 directed against SGG (31), are shown in Fig. 1. The MAb stained only the band corresponding to SGG and did not stain DSGG or the negative control GSL, ceramide trihexoside (Gb_3). The results of experiments to identify DSGG are shown in Fig. 2. In these experiments, the fraction putatively containing DSGG was subjected to a timed, limited desialylation procedure to produce SGG, followed by HPTLC and immunostaining with MAb ID4, directed against SGG (36). A comparison of lanes 1 to 3 in Fig. 2 shows that increasing amounts of SGG are produced over time through desialylation of DSGG, resulting in increasing staining of a band corresponding to SGG on the autoradiograph of MAb ID4 staining shown in Fig. 2A. In the

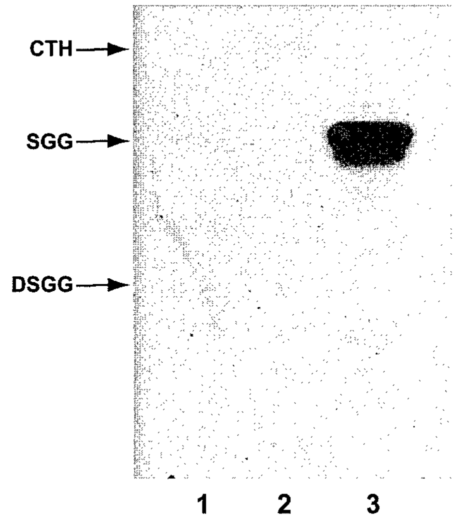

FIG. 1. Identification of SGG purified from human kidney tissue by HPTLC immunostaining. GSL standards, including SGG and DSGG purified from human kidney tissue, were chromatographed and immunostained with MAb RM-1, directed against SGG, as described in Materials and Methods. Lane 1, ceramide trihexoside (Gb3; negative control); lane 2, DSGG; lane 3, SGG.

replicate HPTLC plate stained with orcinol (Fig. 2B), this is reflected by a reduction in orcinol staining of the band corresponding to DSGG, along with an increase in staining of the band corresponding to SGG. At the 7-min desialylation time point, a portion of the sample has likely also been converted to galactosyl globoside, seen as a faint band migrating more rapidly than SGG in lane 3.

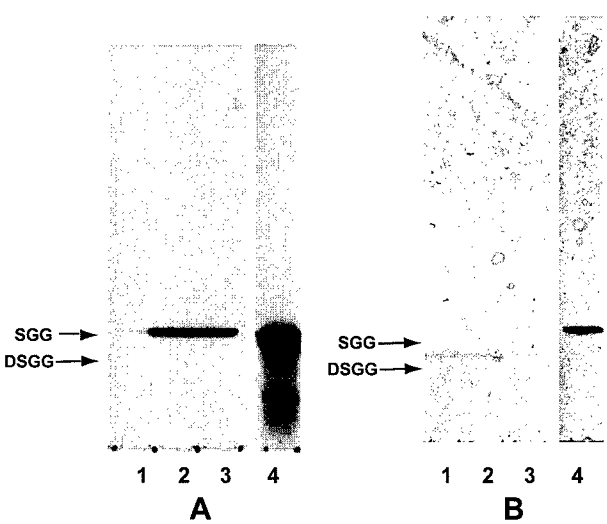

FIG. 2. Identification of DSGG purified from human kidney tissue by timed, limited desialylation of DSGG to SGG, followed by HPTLC immunostaining. DSGG purified from human kidney tissue was identified through desialylation to form SGG, followed by immunostaining. A putative DSGG fraction was subjected to a limited desialylation procedure by incubating aliquots of the sample for 1, 3, and 7 min in 1% acetic acid and then drying the samples, subjecting them to HPTLC, and performing TLC immunostaining with MAb ID4, directed against SGG. (A) Autoradiograph of immunostained HPTLC plate; (B) same HPTLC plate stained with orcinol reagent after the immunostaining procedure. Lanes 1, DSGG fraction after 1 min of desialylation of DSGG; lanes 2, DSGG fraction after 3 min of desialylation; lanes 3, DSGG fraction after 7 min of desialylation; lanes 4, SGG standard.

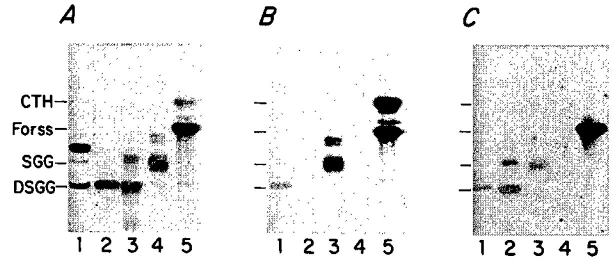

FIG. 3. Binding of representative *E. coli* isolates expressing *pap*-encoded adhesins to SGG and DSGG purified from human kidney tissue. GSLs were purified and separated on HPTLC plates and then overlaid with metabolically [^{35}S]methionine-labeled *E. coli* R45 organisms, which bind globoseries GSLs. Autoradiographs are shown. (A) *E. coli* R45; GSLs: ganglioside fraction (lane 1), DSGG standard (lane 2), DSGG standard isolated from a different preparation and subjected to formal carbohydrate structural analysis (31) (lane 3), SGG standard (note traces of DSGG and galactosyl globoside) (lane 4), and CTH (Gb_3) and Forssman (Forss) standards (lane 5). (B) *E. coli* JJ122; GSLs DSGG standard (lane 1), blank (no GSLs) (lane 2), SGG standard (lane 3), asialo-GM1 (ASGM1) standard (lane 4), and CTH and Forssman standards (lane 5). (C) *E. coli* pJFK102; GSLs: DSGG standard (lane 1), DSGG standard isolated from a different preparation and subjected to formal carbohydrate structural analysis (lane 2), SGG (lane 3), ASGM1 standard (lane 4), and CTH and Forssman standards (lane 5).

Figure 3 shows an example of the multiple bacterial overlay experiments used to purify SGG and DSGG as well as the final result of these purification steps. These experiments demonstrate that SGG and DSGG purified from human kidney tissue bind metabolically ^{35}S-labeled representative *E. coli* isolates R45, JJ122, and pJFK102, expressing P fimbriae carrying *pap*-encoded adhesins of classes II, I, and III, respectively. Nineteen bacterial overlay experiments were performed during the course of purifying SGG and DSGG (12 experiments using *E. coli* R45 and 7 experiments using one or more of the other four *E. coli* isolates described above). We repeatedly observed qualitative differences between the avidities of bacterial binding to SGG and to CTH and other GSL standards with shorter-chain oligosaccharide moieties, based on comparing orcinol staining of known quantities of these purified GSL standards with the amount of SGG in samples still in the process of purification. For example, bacterial binding to 5 to 10 μg of CTH GSL standard was approximately equivalent to the amount of binding to SGG that was at the limits of staining with orcinol, estimated at 20 ng or less. These observations led to the bacterial binding quantitation experiments described below, using purified SGG and DSGG.

Bacterial binding curves. The results of quantitating bacterial binding to serially diluted CTH, globoside, SGG, and DSGG standards both by means of scraping and counting radioactive bands from the silica plates and by performing densitometry of the autoradiographs were essentially identical. Figure 4 shows the autoradiographs from these experiments (left panels) as well as the results of counting radioactivity scraped from bands on silica plates corresponding to binding of metabolically ^{35}S-labeled *E. coli* isolates R45, IA2, pDC1, JJ122, and pJFK102 (right panels). Results of representative experiments are shown for each strain. For each isolate, the relative binding to SGG was greater than the binding to other globoseries GSLs tested. No binding of GSLs by HB101 or in bacterial overlay assays was observed, even when the plates were exposed to film for 7 days (data not shown).

DISCUSSION

In a previous study, we demonstrated that vaginal epithelial cells from nonsecretors selectively express SGG and DSGG

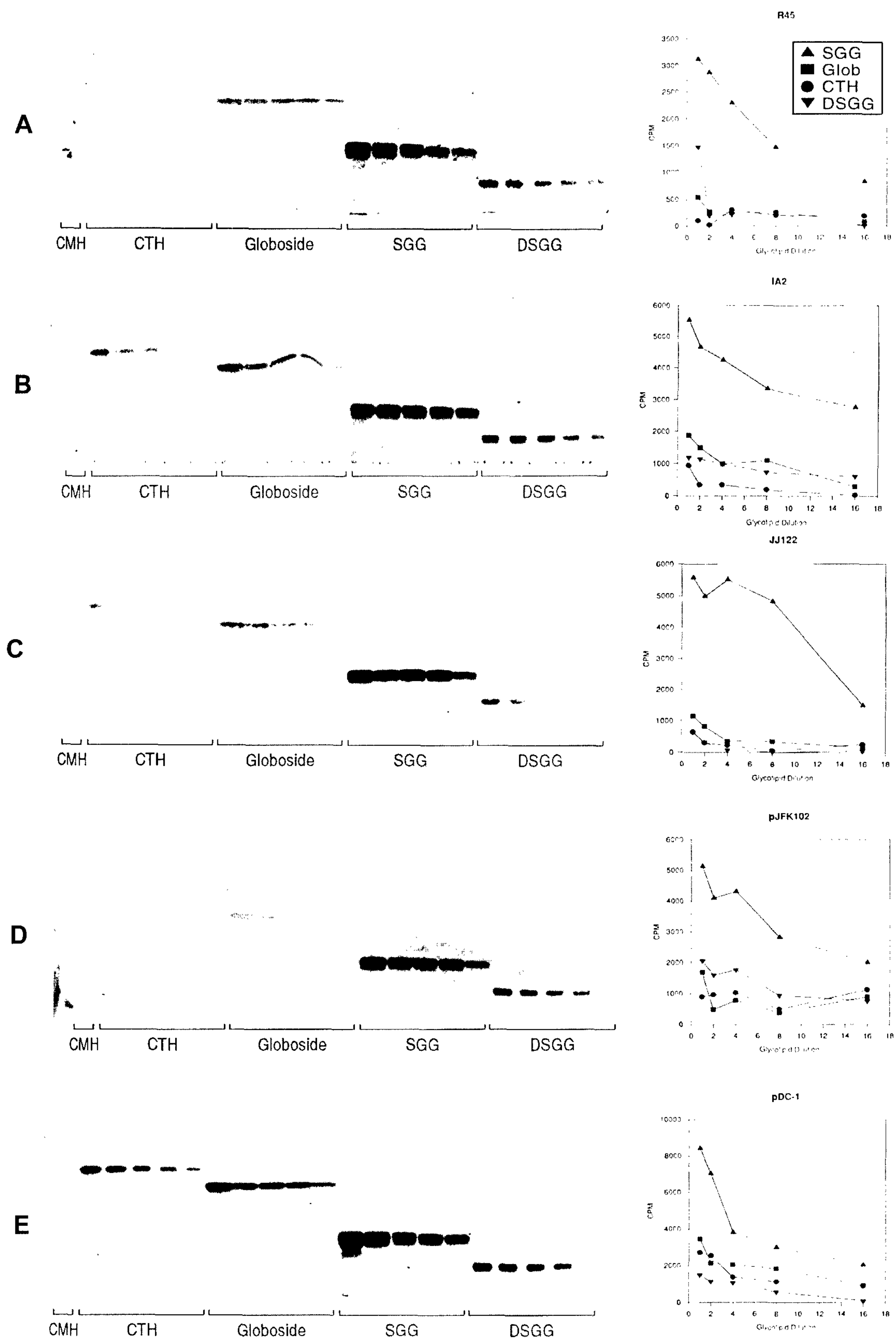

FIG. 4. Binding of representative *E. coli* isolates to serial dilutions of globoseries GSLs in bacterial overlay assays. Autoradiographs of binding are shown in the lefthand panels, and the righthand panels show the quantification of the binding demonstrated in the adjoining autoradiographs. GSLs were serially diluted from 300 to 18.25 ng, chromatographed by HPTLC, and overlaid with representative *E. coli* isolates, as described in the text. The lanes containing standards for CMH (5 μg; negative control), CTH (Gb_3), globoside, SGG, and DSGG are labeled. (A) *E. coli* R45; (B) *E. coli* IA2; (C) *E. coli* JJ122; (D) *E. coli* pJFK102; (E) *E. coli* pDC1. Glob, globoside.

and that these compounds bind a wild-type uropathogenic *E. coli* strain, R45, expressing a *pap*-encoded adhesin (36). Binding did not occur under conditions where *pap*-encoded adhesins were not expressed (36). We reasoned that the presence of these *E. coli*-binding GSLs on the vaginal epithelial cells of nonsecretors but not secretors might explain the increased propensity of nonsecretors for developing recurrent UTIs (5, 17, 32). In the studies reported here, we have now shown that SGG and DSGG are also expressed in human kidney tissues and that these compounds, purified from this source, bind cloned and wild-type uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins. These strains represent the three known classes of P fimbrial adhesins. Using a PCR method that distinguishes the three classes of adhesins (7), we previously determined that *E. coli* R45 expresses P fimbriae carrying a class II adhesin (8, 9). In addition, we demonstrated the binding of SGG and DSGG by IA2, another wild-type isolate expressing P fimbriae carrying a class II *pap*-encoded adhesin, as well as by a cloned isolate expressing this adhesin (PapG from IA2), pDC1 (2). The class I *papG*-encoded adhesin was represented by an isolate expressing P fimbriae carrying PapG from J96 (HB101/pJJ48), expressing the *pap* operon from pHU845 (26), and the class III *papG*-encoded adhesin was represented by pJFK102, which expresses P fimbriae carrying PrsG from J96 (15). Thus, we have demonstrated that SGG and DSGG are relevant bacterial binding moieties for uropathogenic *E. coli* isolates expressing P fimbriae carrying all three known members of the family of *pap*-encoded adhesins.

To investigate the possible biological implications of this finding, we designed experiments to assess the relative binding of these *E. coli* isolates to the GSLs SGG and DSGG (nonsecretor-restricted in the vaginal epithelium [36]) as well as to other relevant globoseries GSLs that we previously identified on both secretors' and nonsecretors' vaginal epithelial cells (36). Before the various classes of *pap*-encoded adhesins were genetically defined, the binding of various wild-type and cloned uropathogenic *E. coli* isolates expressing *pap*-encoded adhesins to globoseries GSLs other than SGG and DSGG was investigated (21, 37). These studies demonstrated relatively little difference between GSL binding to globoside and binding to Gb_3 for those *E. coli* isolates expressing P fimbriae carrying *pap*-encoded adhesins of classes I or II. Isolates expressing P fimbriae carrying a class III *pap*-encoded adhesin demonstrated a preference for binding to extended globoseries GSLs (37). In preliminary experiments, we found that binding of *E. coli* to SGG and DSGG was completely saturated in the GSL concentration range (0 to 1.0 μg) reported in one of these two previous studies, in which a similar technique was used (21, 37). Thus, we constructed GSL binding curves in lower concentration ranges (18 to 300 ng). Although we confirmed most of the previously reported data regarding the relative efficiency of binding of *E. coli* expressing P fimbriae carrying *pap*-encoded adhesin(s) to globoseries GSLs such as Gb_3 and Gb_4, we found that all five *E. coli* isolates bound more strongly to SGG than to the other globoseries GSLs tested, including DSGG. These data demonstrate that, at least in the urogenital epithelia of nonsecretors, SGG may be a preferred ligand for uropathogenic *E. coli* isolates.

In the studies reported here, we have isolated and purified SGG and DSGG from normal human kidney tissue for the first time. Further structural analysis of the SGG sample we obtained from this tissue source by proton nuclear magnetic resonance spectroscopy, mass spectroscopy, and linkage analysis has been completed and will be reported elsewhere (37a), while similar chemical characterization of DSGG from human kidney tissue is ongoing. SGG has been previously isolated, purified, and definitively characterized as to structure only from a human teratocarcinoma cell line, 2102Ep (11). DSGG has been purified from chicken muscle, human erythrocytes, and kidney tumor tissue, and its structure has been definitively proven to be that shown in Table 1 (1, 18, 20, 31). Previous studies by Karr et al. suggested that histological sections of human kidneys could be stained by a MAb directed against stage-specific embryonic antigen 4 (SSEA-4) and that *E. coli* pJFK102 also bound these kidney sections in the same areas stained by the antibodies (15, 16). SSEA-4 is defined as an epitope staining with a MAb raised against 4- and 8-cell-stage mouse embryos and a human teratocarcinoma cell line (33). Based on MAb MC813-70 staining, SSEA-4 has been identified in undifferentiated human embryonic carcinoma cells and seminomas (30). Agglutination of papain-treated human erythrocytes also occurs with MAb MC813-70, identifying the Luke antigen (38, 39), but the molecule on which the Luke antigen is carried on erythrocytes has not been isolated and structurally characterized. Thus, the antibody staining data previously reported by Karr et al. suggested, but did not prove, that SGG was expressed in human kidney tissue. Our data unequivocally demonstrate the presence of both SGG and DSGG in human kidney tissue.

In conclusion, our studies demonstrate the presence of SGG and DSGG in the human kidney and define SGG as a GSL to which each of the three classes of *pap*-encoded adhesins binds avidly. The biological significance of these findings requires further study, but since *E. coli* isolates bearing P fimbrial adhesins are very strongly associated with renal infection (6), SGG may well play a role in the pathogenesis of acute pyelonephritis. Svanborg and associates have also reported an association between nonsecretor status and an increased likelihood of clinically defined inflammatory responses suggestive of pyelonephritis, such as fever, leukocytosis, and elevated C-reactive protein (23). The presence of SGG in the kidneys of nonsecretors could play a role in predisposing these patients to renal inflammation. Further studies are needed to more extensively define the expression of SGG and DSGG in epithelial tissues throughout the urogenital tract. Our data demonstrate the presence of these compounds in the vagina (36) and kidney; we are currently studying the GSL composition of normal human bladder epithelium, including assaying for the presence of SGG and DSGG. Data derived from these studies will increase our knowledge of bladder glycobiology and may eventually lead to novel preventive strategies for UTI through the use of carbohydrate-based compounds that competitively inhibit bacterial attachment.

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The P Histo-Blood Group-Related Glycosphingolipid Sialosyl Galactosyl Globoside as a Preferred Binding Receptor for Uropathogenic *Escherichia coli*: Isolation and Structural Characterization from Human Kidney†

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ABSTRACT: The P histo-blood group-related glycosphingolipid, sialosyl galactosyl globoside (SGG), has recently been implicated as a preferred binding receptor for uropathogenic *Escherichia coli* [Stapleton, A. E., Stroud, M. R., Hakomori, S., and Stamm, W. E. (1998) *Infect. Immun. 66*, 3856–3861]. We report here the purification and complete structural characterization of SGG from normal human kidney. Using metabolically [^{35}S]-labeled *E. coli* as a probe, a monosialylated glycosphingolipid was isolated to homogeneity. The glycosphingolipid was purified by a combination of high-performance liquid chromatography and preparative high-performance thin-layer chromatography and its structure unambiguously elucidated by ^{1}H NMR, electrospray ionization mass spectrometry, and methylation analysis. Its primary structure was shown to be identical to a previously characterized, developmentally regulated, globo-series glycolipid thought to be unique to human teratocarcinoma. The significance of this structure as a unique receptor in human kidney for uropathogenic *E. coli* and its role in the pathogenesis of urinary tract infections are discussed.

Nearly all of the antigenic determinants defining the P histo-blood group system (P^k, P, LKE, and P$_1$) are associated with globo-series glycosphingolipids (*1, 2*). Globo-series glycosphingolipids (GSLs) are defined at the chemical level as having the trisaccharide Galα1→4Galβ1→4Glcβ1 (P^k) attached at the reducing end to ceramide (*N*-fatty acyl sphingosine) (Table 1). This "globo-core" can be further glycosylated to form more complex carbohydrate structures that define additional P blood group related antigens, i.e., P and LKE (*3*), as well as those antigens associated with the ABO histo-blood group system (*4*). P blood group antigenic structures are strictly carried on glycosphingolipids (*5*) and play an important role in the pathogenesis of urinary tract infections, particularly as receptors for uropathogenic *Escherichia coli* (*6*). The Galα1→4Gal sequence found in all blood group P antigens is the minimal structure required for binding P fimbria-expressing uropathogenic *E. coli* (*7, 8*). However, women expressing P blood group related antigens who carry ABH blood group antigens in secretions (secretors) have a lower incidence of urinary tract infections (UTI)[1] than individuals (nonsecretors) who do not express these determinants (*9–11*). It has been hypothesized that the increased risk of UTI in nonsecretors and the increased attachment of uropathogenic bacteria to their uroepithelial cells (*11*) is due to the expression of a unique receptor for *E. coli* (*12*). Evidence suggests that nonsecretors selectively express two extended globo-series GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG), which specifically bind uropathogenic *E. coli* (*12*), and that one of these GSLs (SGG) is the preferred receptor over other globo-series GSLs for *E. coli* isolates expressing a P-related adhesin (*13*).

Since globo-series glycolipids have been shown to be modified by histo-blood group status, and the globo-core is the major carrier isotype (type 4 chain) of ABH active epitopes in renal epithelium (*46*), we hypothesized that the increased risk of UTI in nonsecretors may be due to the presence of a unique receptor for P-fimbriated *E. coli* in these patients. In the present study, using a metabolically [^{35}S]-labeled clinical *E. coli* isolate (R45) as a probe, a monosialylated ganglioside comigrating with a sialosyl galactosyl globoside standard by high-performance thin-layer chromatography (HPTLC) in two different solvent systems was purified from normal human kidney. Its structure was

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[1] Abbreviations: Cer, ceramide; C/M, chloroform/methanol; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; ^{1}H NMR, proton nuclear magnetic resonance; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; IHW, isopropyl alcohol–hexane–water; MAb, monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; SGG, sialosyl galactosyl globoside; SSEA-4, stage specific embryonic antigen 4; UTI, urinary tract infection. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Lipids* (1977) *12*, 455–463]; however the suffix –OseCer is omitted.

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Table 1: Structures of Glycosphingolipids Referred to in This Study[a]

glycosphingolipid	structure	P blood group activity
CTH	Galα1−4Galβ1−4Glcβ1−1Cer	P^k
Globoside	GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	P
Gal-globoside	Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Forssman	GalNAcα1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Globo-H	Fucα1−2Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
Globo-A	GalNAcα1−3(Fucα1−2)Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	
P^1	Galα1−4Galβ1−4GlcNAcβ1−3Galβ1−4Glcβ1−1Cer	P_1
SGG	NeuAcα2−3Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	LKE
DSGG	NeuAcα2−3Galβ1−3(NeuAcα2−6)GalNAcβ1−3Galα1−4Galβ1−4Glcβ1−1Cer	

[a] Key: CTH, ceramide trihexoside (Gb_3, globotriaosylceramide); globoside, Gb_4 (globotetraosylceramide); gal-globoside, galactosyl globoside, Gb_5, SSEA3; SGG, sialosyl galactosyl globoside, SSEA4; DSGG, disialosyl galactosyl globoside; Cer, ceramide; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid.

unambiguously confirmed by ^{1}H NMR, electrospray ionization mass spectrometry (ESI-MS), and methylation analysis as $V^3NeuAcGb_5Cer$ (SGG), a developmentally regulated globo-series glycosphingolipid previously thought to be unique to human teratocarcinoma and sharing the same terminal epitope as the stage-specific embryonic antigen, SSEA-4. To our knowledge, this is the first report describing the complete and unambiguous structural characterization of SGG from normal human tissue. Its identification in human kidney, a target organ for uropathogenic *E. coli* infection, and its role as a high affinity ligand (*13*) may explain the chemical basis for the increased risk of UTI in nonsecretors.

MATERIALS AND METHODS

Preparation of Human Kidney Ganglioside Fraction. (A) Glycolipid Extraction. Approximately 800 g of pooled human kidney tissue was extracted by homogenization (*14*) in a Waring blender with 10 volumes of the lower phase of 2-propanol:hexane:water (IHW; 55:25:20). The extract was filtered through a Whatmann No. 1 filter and the residue reextracted and filtered as above. The extraction/filtration procedure was repeated once more, and the combined filtrates were concentrated under reduced pressure at 40 °C using a Brinkman rotary evaporator. The concentrated extract was subjected to Folch partitioning by dissolving the residue in ? 3 L of C/M (2:1) containing 500 mL of water. After vigorous shaking, the extract was allowed to separate until two translucent phases appeared (*15*). The upper phase was removed and the lower phase reextracted by the addition of C/M/1% KCl (1:10:10) to the original level. The liquid−liquid extraction procedure was repeated two times, and the combined upper phases were concentrated by rotary evaporation, reconstituted in water, and dialyzed exhaustively against deionized water using Spectropor 3 dialysis tubing (MW cutoff = 3500).

(B) Anion Exchange Chromatography. After dialysis, the upper-phase extract was evaporated to dryness as above and dissolved in 50 mL of C/M/water (30:60:8) by a combination of warming at 37 °C and sonication. Insoluble material was removed by centrifugation at 1000g for 10 min and reextracted by sonication in an additional 50 mL of the same solvent. Following centrifugation as above, the combined supernatants were loaded onto a DEAE-sephadex column (300 mL bed volume; acetate form) and washed with 2 L of C/M/water (30:60:8) to remove all neutral lipids (*16*). The column was equilibrated with 500 mL of methanol and the ganglioside fraction eluted with 2 L of 1.0 M NH_4OAc in methanol. The eluted ganglioside fraction was dried by rotary evaporation, dialyzed against water, and dried as above.

Purification of Sialosyl Galactosyl Globoside from Human Kidney Ganglioside Fraction. (A) High-Performance Liquid Chromatography. The ganglioside fraction was solubilized in 10 mL of IHW and transferred from the evaporation flask to a 15 mL tube. The sample was completely dried under N_2 at 37 °C using a nitrogen evaporator (N-EVAP, Organomation Inc., South Berlin, MA) and reconstituted in 2 mL of IHW by sonication. The sample was injected onto a preparative Iatrobead column (6RS-8010; 0.8 × 60 cm; Iatron Laboratories Inc., Kanda/Tokyo, Japan) preequilibrated with IHW (55:40:5), and subjected to a linear gradient from IHW 55:40:5 to 55:25:20 with a flow rate of 1 mL/min (*17*). Fractions of 4 mL each were collected over 400 min. Each fraction was spotted onto an HPTLC plate, developed in chloroform/methanol/0.5% $CaCl_2$ (described below), and visualized by spraying with 0.5% orcinol in 2 N sulfuric acid. A parallel plate was developed and used in bacterial overlay assays (see below). Fractions staining positive to orcinol and *E. coli* were pooled according to migration. HPLC fractions 39−48, showing the strongest binding by the *E. coli* overlay assay and containing more than one band by TLC, were pooled, dried under N_2, resolubilized in 1 mL of IHW, and injected onto a semipreparative Iatrobead column (0.4 × 60 cm). A linear gradient from IHW 55:40:5 to 55:25:20 over 200 min with a flow rate of 0.5 mL/min was used. Fractions of 1 mL each were collected, assayed, and pooled as described above. One pool (fractions 53−58), showing the strongest staining by *E. coli* and containing multiple bands by TLC, was further purified by preparative HPTLC (described below).

(B) High-Performance TLC. The strongest staining band in fraction 53−58 was separated by preparative HPTLC in chloroform/methanol/0.5% $CaCl_2$ (50:40:10) so only a single orcinol and *E. coli* positive band was observed. The sample was further resolved into three additional bands by HPTLC using a solvent system of 1-propanol/water/NH_4OH (6:1.5:1). Preparative TLC was performed by streaking 50 μL of sample across a 10 × 20 cm HPTLC silica gel plate (silica gel 60; EM Science, Gibbstown, NJ), drying, and developing in the appropriate solvent system. Plates were dried, and bands were visualized by spraying with 0.03% primulin in 80% acetone. Bands were marked with a pencil under UV light. Marked bands were scraped from the plate using a razor blade, and the gangliosides were extracted from the silica

by sonicating for 20 min in IHW (55:25:20; 2 mL per band). The silica was removed by centrifuging at 1000g for 10 min and reextracted as above, and the combined supernatants were dried under N_2. Samples were cleaned up using 1 cm^3 tC-18 Sep-Pak cartridges (Waters, Milford, MA) by first dissolving the sample in 1 mL of PBS and then applying it to a column equilibrated with PBS after sequentially washing with 5 mL of methanol and 5 mL of water. Once the sample was retained, the column was washed with 10 mL of water, followed by 10 mL of 50% methanol, and eluted in 5 mL of 100% methanol. The sample was dried under N_2, dissolved in 1 mL of IHW (55:25:20), and injected onto an Iatrobead column (0.4 × 30 cm) as above using a linear gradient from IHW 55:40:5 to 55:25:20 for 100 min at a flow rate of 1 mL/min. Fractions of 1 mL each were collected and visualized by HPTLC using the orcinol–sulfuric acid reaction. Orcinol–positive fractions were pooled and dried under N_2 prior to structural analysis.

Bacterial Overlay Assays. Assays were performed as previously described (*12*) using metabolically [^{35}S]-labeled *E. coli* isolate R45, a wild-type cystitis isolate (*18*), which expresses P fimbriae carrying the class II *pap*-encoded adhesin (*19*) and binds globo-series glycolipids.

^{1}H-Nuclear Magnetic Resonance Spectroscopy. A sample of the ganglioside was prepared for NMR analysis by repeated lyophilization from D_2O (99.996 at. %; Cambridge Isotope Laboratories, Woburn, MA) and then dissolved in 0.5 mL DMSO-d_6 (99.96 atom %; Aldrich, Milwaukee, WI) containing 2% D_2O. All ^{1}H NMR spectra were acquired at 600 MHz on a Bruker (Karlsruhe, Germany) DRX-600 Fourier transform spectrometer, at a probe temperature of 308 K and a sweep width of 3600 Hz and with suppression of the residual HOD resonance by a presaturation pulse during the preparatory delay period. One-dimensional ^{1}H NMR spectra were resolution enhanced by exponential multiplication (LB = 0.3) prior to Fourier transformation. Acquisition and processing of 2-D TPPI–COSY, –TOCSY, and –NOESY experiments were performed essentially as described previously (*20*, and references cited therein).

Positive Ion Mode Electrospray Ionization Mass Spectrometry. ESI-MS experiments were carried out on a PE-Sciex API–III spectrometer with IonSpray source. A sample of ganglioside was permethylated as described previously (*21*), dissolved in methanol containing 1 mM ammonium acetate, and introduced by direct infusion. For single quadrupole spectra, the mass range m/z 100–2200 was scanned at an orifice-to-skimmer potential of 180 V, or, for a higher resolution spectrum of the pseudomolecular ion region, m/z 2000–2250 at 200 V. For tandem ESI-MS/CID-MS experiments, the orifice-to-skimmer potential was lowered to 120 V to increase the abundance of disodiated, doubly charged pseudomolecular ions; argon was introduced into the collision cell at CGT ≅ 400, and precursor ions were selected in Q_1, while the mass range m/z 100–2200 was scanned in Q_3.

Linkage Analysis by GC-MS. An aliquot of permethylated ganglioside was depolymerized, reduced, and acetylated essentially as described (*21*). Analysis of the resultant partially methylated alditol acetates (PMAAs) was performed on a Hewlett-Packard 5890 GC/5970 MSD operating in the splitless mode, using a 30 m DB-5 bonded phase fused silica capillary column, temperature programmed from 160 to 260 °C at 2 °C/min. PMAA derivatives were identified by retention times and characteristic EI mass spectra (*22*, *23*) compared with those of authentic standards.

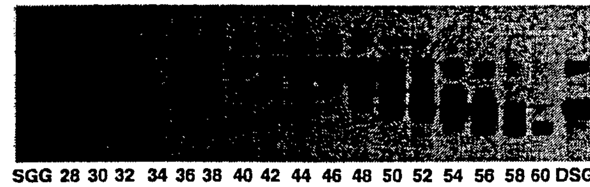


FIGURE 1: HPTLC of ganglioside fractions of normal human kidney separated by HPLC in CMW solvent system 50:40:10. Total ganglioside fraction from human kidney was separated by normal phase preparative HPLC using a linear gradient from IHW 55:40:5 to 55:25:20. Fractions were collected and separated by HPTLC in CMW (50:40:10). Bands were revealed by overlaying HPTLC plate with metabolically [^{35}S]methionine-labeled *E. coli* isolate R45. SGG, sialosyl galactosyl globoside standard; DSGG, disialosyl galactosyl globoside standard. Fractions 39–48, comigrating with SGG standard was pooled and subjected to a second HPLC (semipreparative HPLC, see text). Autoradiograph is shown.

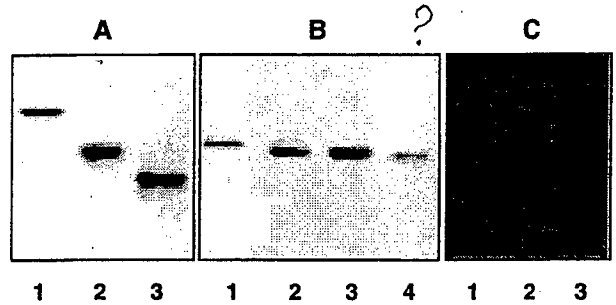


FIGURE 2: HPTLC of purified gangliosides from human kidney. HPTLC plates of three unknown gangliosides purified from pooled HPLC fraction 53–58 (from semipreparative HPLC). Panel A, pooled ganglioside fraction (53–58) previously migrating as a single band in CMW system is resolved into three bands using a solvent system of 1-propanol/water/NH_4OH (6:1.5:1) after preparative TLC in the same solvent system; stained with orcinol/sulfuric acid reagent. Panel B, the same three gangliosides shown in panel A developed in CMW (50:40:10) and stained with orcinol/sulfuric acid reagent. Panel C, autoradiograph of HPTLC plate identical to the samples and solvent conditions used in panel B stained with [^{35}S]-labeled *E. coli* isolate R45. Ganglioside fraction 3 (lane 3) showing strong staining with *E. coli* was structurally characterized.

RESULTS

Purification of SGG from Normal Human Kidney. The HPLC elution pattern of *E. coli*-binding gangliosides as determined by HPTLC is shown in Figure 1. Fractions 39–48 were pooled on the basis of strong binding to [^{35}S]-labeled *E. coli* and comigration with an SGG standard prepared from partially desialylated DSGG. Pooled fraction 39–48 was subjected to a second HPLC as described in Materials and Methods. Fractions 53–58 obtained after the second HPLC were pooled on the basis of the same criteria described above. A single band comigrating with the SGG standard in C/M/0.5%$CaCl_2$ (50:40:10) was obtained after preparative HPTLC in the same solvent system (data not shown). The GSL sample was further resolved into three components and purified by preparative TLC in a solvent system consisting of 1-propanol/water/NH_4OH (6:1.5:1). A thin-layer chromatogram showing the separation of the three GSL components after preparative TLC in the above solvent system is shown in Figure 2A. The three GSL fractions were labeled according to their migration in 1-propanol/water/NH_4OH (6:

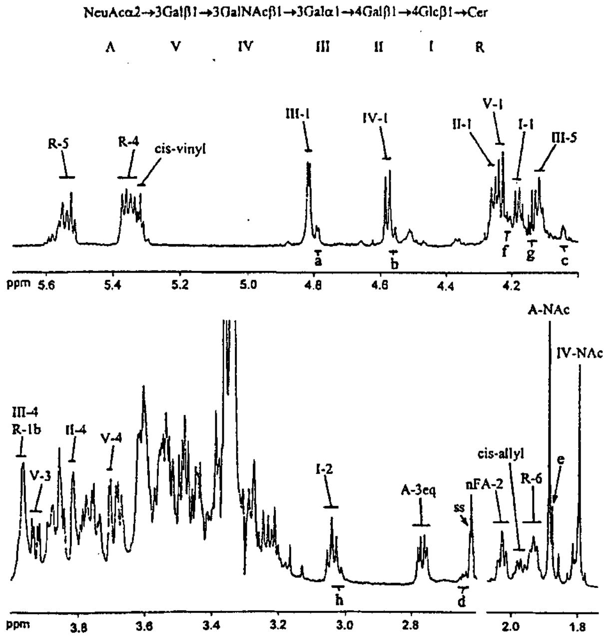


FIGURE 3: Selected regions of a 1-D proton NMR spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D_2O at 308 K. Region from 1.7 to 2.1 ppm is attenuated ×0.4. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure drawn at the top of the figure. R refers to protons of the sphingosine backbone only; cis-vinyl and cis-allyl refer to protons of unsaturated *N*-fatty acyl chains; nFA-2 refers to H-2 of non-hydroxylated *N*-fatty acyl chains. Resonances from minor components are designated by small letters and assigned as follows: a−e, III-1, IV-1, III-4, A-3eq, and B-NAc, respectively, of a monosialoganglioside component proposed to have an isomeric structure with NeuAc (B) linked α2→6 to *β*-GalNAc IV of galactosylgloboside; f and h, I-1 and I-2 of ganglioside component with 2-hydroxy fatty acyl chains; g, unknown impurity.

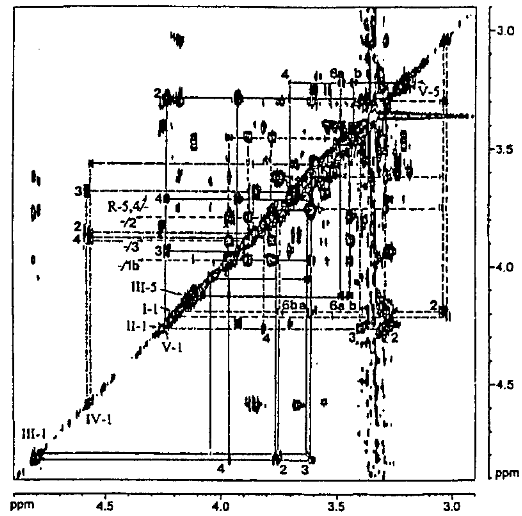


FIGURE 4: Downfield region of 2-D TOCSY spectrum of the monosialosyl ganglioside from human kidney in DMSO-d_6/D_2O at 308 K. Monosaccharide residue H-1 and selected H-5 resonances are designated on diagonal by Roman and Arabic numerals as in Figure 3. Off-diagonal correlations are marked by Arabic numerals only. Upper left section: connectivities originating from V-1 and V-5 (solid lines), R-5 and R-4 (dashed lines), and IV-1 (dashed/dotted lines). Lower right section: connectivities originating from III-1 and III-5 (solid lines), I-1 (dashed lines), and II-1 (dashed/dotted lines). Connectivities originating from minor components are shown for III-1, IV-1, and I-1, resonances a, b, and f, respectively in Figure 3.

1.5:1); i.e., the fastest migrating component was labeled 1 and the slowest 3. All three samples were rechromatographed in C/M/0.5%$CaCl_2$ (50:40:10) on duplicate plates. Figure 2B shows a thin-layer chromatogram stained for carbohydrates by the orcinol−sulfuric acid reaction. Figure 2C shows the same chromatogram as in Figure 2B with the bands revealed by [^{35}S]-labeled *E. coli.* Fraction 3, which comigrated with the SGG standard (Figure 2B) and showed the strongest binding by *E. coli* (Figure 2C), was subjected to structural analysis.

^{1}H-NMR Spectroscopy. Portions of the 1-D ^{1}H NMR spectrum of the monosialosyl ganglioside from human kidney are reproduced in Figure 3. Although a number of signals from glycosphingolipid and other impurities can be observed, the spectrum of the major component is clearly similar to that obtained previously from V^{3}NeuAcGb$_5$Cer (GL-7)

Table 2: Proton Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) for Sialosylgalactosylgloboside in Dimethyl Sulfoxide-d_6/2% D_2O at 308 K

	NeuAcα2⟶3Galβ1⟶3GalNAcβ1⟶3Galα1⟶4Galβ1⟶4Glcβ1⟶1Cer						
	A	V	IV	III	II	I	R
H-1		4.234	4.579	4.815	4.256	4.185	3.447 (a)
($^3J_{1,2}$)		(7.9)	(8.3)	(4.2)	(7.5)	(7.7)	3.962 (b)
H-2		3.279	3.858	3.766	3.310	3.040	3.781
H-3	2.766 (eq) 1.346 (ax)	3.926	3.675	3.609	3.399		3.880
H-4	3.538	3.706	3.880	3.964	3.815		5.354
H-5				4.117		3.290	5.538
H-6				3.476 (a) 3.454 (b)		3.611 (a) 3.747 (b)	1.935
Nac	1.889		1.795				

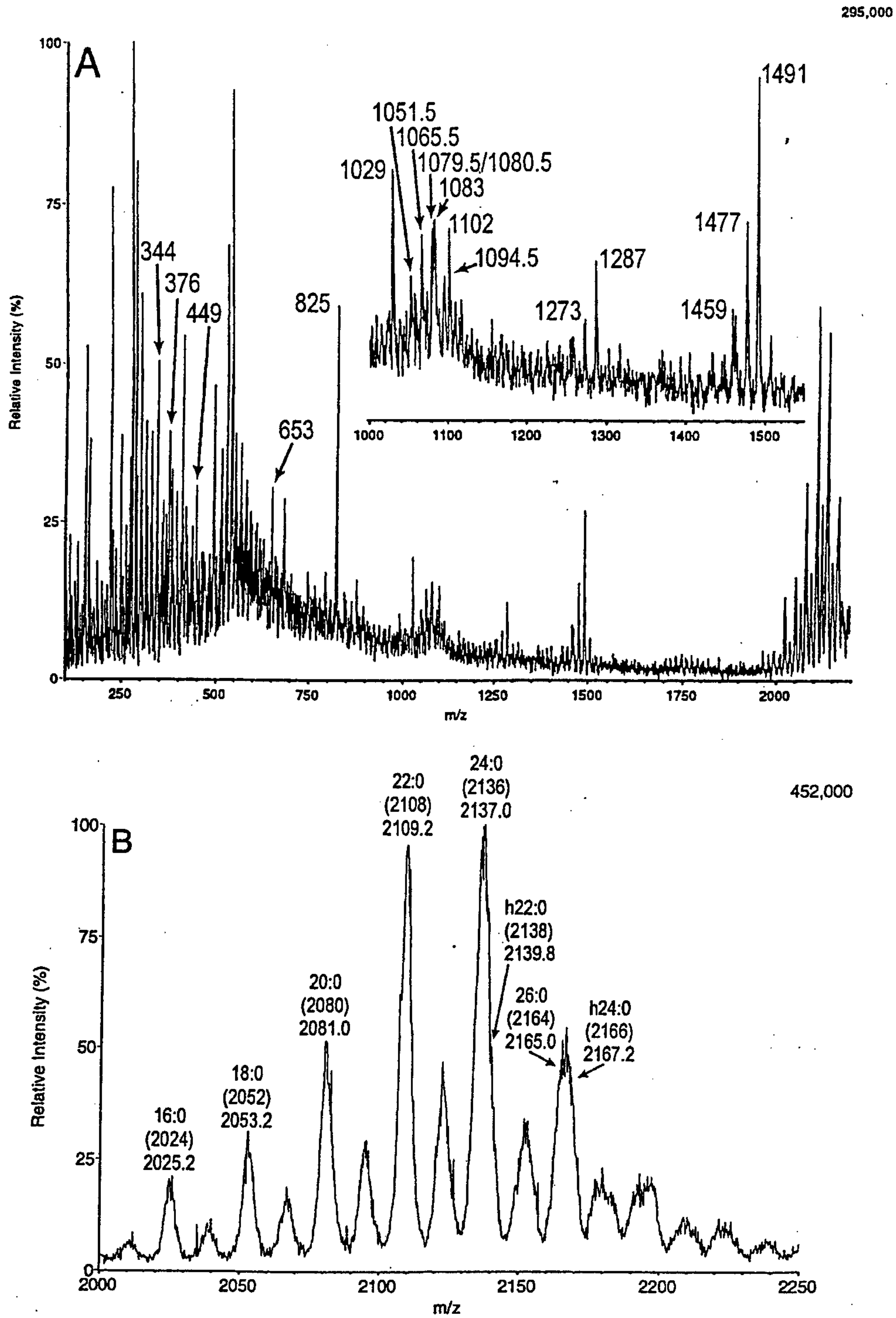


FIGURE 5: Positive ion mode ESI-CID-MS (orifice-to-skimmer potential = 180 V) of permethylated monosialosyl ganglioside from human kidney. Panel A, mass range *m/z* 100–2200. Inset, expansion of *m/z* 1000–1550; relevant fragments are designated by nominal monoisotopic mass (see Scheme 1). Panel B, mass range *m/z* 2000–2250 scanned at higher resolution; monosodiated, singly charged, pseudomolecular ions are designated by fatty acid species (top), nominal monoisotopic mass (parentheses), and measured *m/z* (to nearest 0.1 u).

originally isolated from the human teratocarcinoma cell line 2102Ep (*24*). With respect to anomeric and other structural reporter resonances, the chemical shift differences are all <0.01 ppm. The sugar residues of the core glycan are represented by five anomeric resonances, four β ($^3J_{1,2} = 7-9$ Hz) and one α ($^3J_{1,2} = 2-4$ Hz), as expected for a Gb_5 pentasaccharide. The chemical shifts of two β-anomeric signals (4.185 and 4.256 ppm) and the single α-anomeric signal (4.815 ppm) are very close to those of β-Glc I, β-Gal II, and α-Gal III, respectively, in Gb_4Cer and Gb_5Cer measured under similar conditions (*24, 25*). The remaining β-anomeric resonances (4.579 and 4.234 ppm) correspond to those for β-GalNAc IV and β-Gal V, respectively, in Gb_5Cer, with a relative upfield shift for the former (−0.03 ppm) and a downfield shift for the latter (+0.04 ppm). These are the glycosylation-induced shift changes expected for

addition of NeuAcα2→3 to a terminal Galβ1→3/4HexNAc group (*26, 27*). The additional NeuAc residue is distinguished by an H-3eq signal (2.766 ppm, dd) and an NAc signal (1.889 ppm, s, 3H) at chemical shifts diagnostic for an α2→3 linkage to terminal β-Gal (*26, 27*). A second NAc signal (1.795 ppm, s, 3H) could be assigned to the β-GalNAc residue; it was observed previously at 1.797 ppm in the spectrum of $V^3NeuAcGb_5Cer$ (*28*).

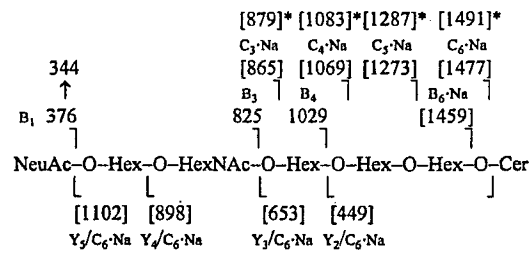
To substantiate the proposed glycan structure, as well as confirm the proton resonance assignments, the sample was subjected to a series of 2-D NMR experiments, including PS-DQF-COSY, TOCSY (see Figure 4), and NOESY. This allowed assignment of H-1 through H-4 of all three β-*galacto*pyranoside spin systems, H-1 through H-6 of both the α-*galacto*pyranoside and the β-*gluco*pyranoside spin systems, and H-3 through H-5 of the NeuAc spin system, along with the functionalized proximal part of the ceramide spin system. Because of the vanishingly small $^3J_{4,5}$ coupling in galactopyranosides, which attenuates transmission of coherence between H-4 and H-5 of a *galacto*pyranoside (*29–31*), it was not practical to assign H-5 and H-6 resonances for these spin systems, except in the case of α-Gal III, where the distinct downfield position of H-5 allows a connectivity to be established with the remaining ring protons in the TOCSY experiment. The assignments, the majority of which have not been available previously for this compound, are summarized in Table 2. The glycan primary structure was further substantiated by detection of the following dipolar interactions between glycosidically linked residues in the NOESY experiment: V-1/IV-3; IV-1/III-3; III-1/II-4; and II-1/I-4. Although no interglycosidic dipolar interactions were detected originating from the NeuAc residue, the NeuAcα2→3 linkage to terminal β-Gal was confirmed by the diagnostic positions observed for V-3, V-4, and A-3ax (*26, 27*).

The complex of resonances corresponding to the major ceramide component indicated that it is composed of sphing-4-enine and non-α-hydroxylated fatty acids (*26, 32*). Only a small amount of fatty acid unsaturation is indicated by the low amplitudes of the cis-vinyl proton signal at 5.320 ppm and the allyl proton signal at 1.976 ppm. The presence of a ceramide component containing α-hydroxylated fatty acids is indicated by the observation of additional β-Glc H-1 and H-2 resonances at 4.215 and 3.026 ppm, respectively (*27*).

Permethylation and ESI-MS. Following permethylation, a portion of the material was subjected to analysis by electrospray ionization mass spectrometry in the positive ion mode. Single quadrupole analysis at high orifice-to-skimmer potentials (150–200 V) gave abundant singly charged $[M\cdot Na]^+$ and doubly charged $[M\cdot 2Na]^{2+}$ pseudomolecular ion species. The overall abundances, as well as the ratio, of these species depended on the potential used, although they were not always strictly reproducible even at a given potential due to the influence of other variables. High potentials resulted in significant and useful glycosidic fragmentation, yielding ions of the B- and [C·Na]-type, along with some of the [B·Na]- and [Y/C·Na]-type ions[2] normally seen under ESI-MS/CID-MS conditions. An additional set of ions corresponding to $[C\cdot Na + CH_2]$ were observed for a number of glycosidic cleavages; the precise origin of these fragments will be discussed elsewhere.[3]

Scheme 1: Prominent Pseudomolecular Ions and Fragmentation of Permethylated Hexaglycosylceramide in ESI-MS at High Orifice-to-Skimmer Potentials[a]

fa	Cer	MW	$M\cdot Na^+$	$M\cdot 2Na^{2+}$
16:0	548	2001	[2024]	[[1023.5]]
18:0	576	2029	[2052]	[[1037.5]]
20:0	604	2057	[2080]	[[1051.5]]
22:0	632	2085	[2108]	[[1065.5]]
24:0	660	2113	[2136]	[[1079.5]]
26:0	688	2141	[2164]	[[1093.5]]
h22:0	662	2115	[2138]	[[1080.5]]
h24:0	690	2143	[2166]	[[1094.5]]



[a] Key: [ ] = monosodiated monocation; [ ]* = monosodiated monocation + 14 u; [[ ]] = disodiated dication. All values are nominal monoisotopic masses. Fa = fatty acyl group.

The monosodiated pseudomolecular ion series (see Figure 5; Scheme 1) corresponded to a glycan formula $NeuAc\cdot Hex_4\cdot HexNAc$ attached to ceramides consisting of d18:1 sphingosine in combination with fatty acids 16:0–24:0, with 22:0 and 24:0 predominating. The presence of significant amounts of h22:0 and h24:0 fatty acids was also apparent from pseudomolecular ions 30 u higher in mass (in the former case, the ion at *m/z* 2138 is poorly resolved from that at *m/z* 2136). The fragmentation clearly supports the linear glycan sequence proposed for the ganglioside, as shown in Scheme 1. Although the lack of an observable B_2 ion at *m/z* 580 means that the NeuAc residue cannot be definitively placed on the terminal Hex on the basis of this series alone, the combination of internal glycan ions $[Y_5/C_6\cdot Na]$ (*m/z* 1102) and $[Y_4/C_6\cdot Na]$ (*m/z* 898) is supportive. However, the latter ion is of rather low abundance. ESI-MS/CID-MS experiments, as described below, were more definitive.

Triple quadrupole ESI-MS/CID-MS experiments were carried out under conditions maximizing the yield of disodiated doubly charged pseudomolecular ions. The most abundant of these were selected by Q_1 and collisionally activated in Q_2 while scanning Q_3. The results of three of these experiments are reproduced in Figure 6. The spectra are characterized by an abundance of glycosidic cleavage fragments of the [B·Na]-, [C·Na]-, [Y·Na]- [Y/B·Na]-, and [Y/C·Na]-types (see Scheme 2). Of particular significance is the abundant ion *m/z* 620, corresponding to the $[C_2\cdot Na]$ fragment which clearly shows the attachment of NeuAc to the terminal Hex residue.

Linkage Analysis by GC-MS. The remainder of the permethylated ganglioside was depolymerized, reduced, and acetylated according to standard procedures, and the resulting partially methylated alditol acetates (PMAAs) analyzed by

[2] The nomenclature of Domon and Costello (*33*) is used here.

[3] Levery, S. B. (manuscript in preparation).

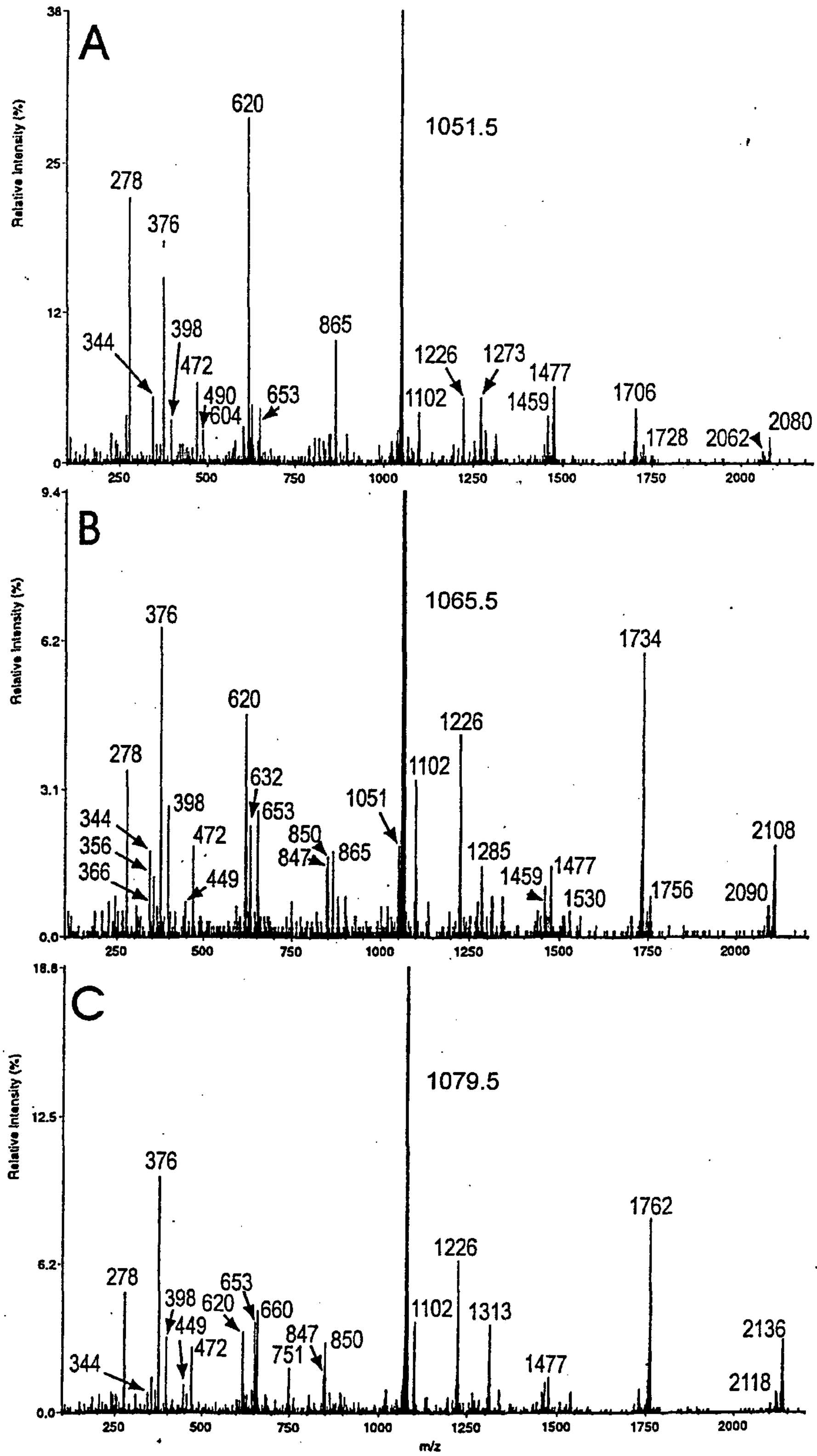
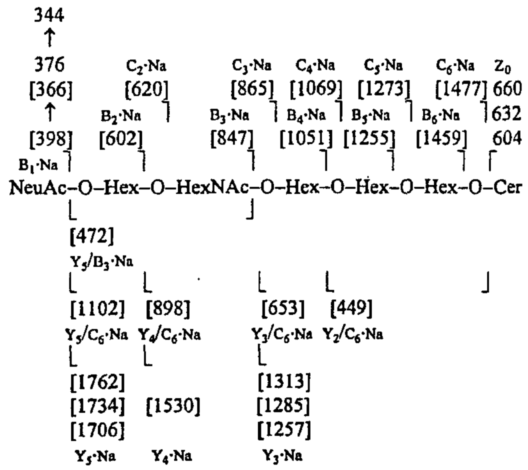


FIGURE 6: Product ion spectra from ESI-MS/CID-MS (orifice-to-skimmer potential = 120 V) of selected doubly charged disodiated pseudomolecular ions of permethylated monosialosyl ganglioside from human kidney. The orifice-to-skimmer potential was lowered to increase abundance of doubly charged disodiated pseudomolecular ions. Panel A, Q_1 selection of *m/z* 1051.5. Panel B, Q_1 selection of *m/z* 1065.5. Panel C, Q_1 selection of *m/z* 1079.5. Relevant fragments are designated by nominal monoisotopic mass (see Scheme 2).

GC-EI-MS. PMAAs identified by their retention times and characteristic EI spectra were 2,3,6-tri-O-Me-Gal (→4Gal); 2,4,6-tri-O-Me-Gal (→3Gal); 2,3,6-tri-O-Me-Glc (→4Glc); and 4,6-di-O-Me-GalNAcMe (→3GalNAc). Detection of

Scheme 2: Fragmentation of Q_1-Selected Doubly Charged Disodiated Pseudomolecular Ions $M \cdot 2Na^{2+}$ of Permethylated Hexaglycosylceramide in ESI-MS/CID-MS[a]

fa	M•2Na^{2+}	M•Na^{+}	Cer
20:0	[[1051.5]]	[2080]	604
22:0	[[1065.5]]	[2108]	632
24:0	[[1079.5]]	[2136]	660



[a] Key: [ ] = monosodiated monocation. All values are nominal monoisotopic masses. Fa = fatty acyl group.

these derivatives confirmed unambiguously all of the linkages proposed on the basis of the NMR analysis, and, together with the residue sequence derived from ESI-MS data, confirms that the complete primary structure is V^3NeuAcGb$_5$Cer.

DISCUSSION

Globo-series glycosphingolipids are characterized by an internal Galα1→4Gal sequence and are found in the outer leaflet of the plasma membrane where they are exposed to the extracellular environment (*35*). The expression of globo-series glycolipids is determined by the P histo-blood group system (*1, 2*) and plays an important role in the pathogenesis of UTI by mediating the attachment of *E. coli* to uroepithelial cells. Uropathogenic *E. coli* bind to P blood group antigens via *pap* gene encoded fimbrial adhesins, hence the name P fimbriae (*6, 36*). In addition to the role of P blood group antigens in the pathogenesis of UTI, they have also been shown to act as receptors for verotoxins (*37, 38*) and parvoviruses (*39*), and antibodies directed against some of these antigens (P and P^k) have been implicated in spontaneous abortion (*40–42*) as well as the rare autoimmune disorder paroxysmal cold hemoglobinuria (*43*).

SGG is a sialylated extended form of globo-series glycolipid first reported as a developmentally regulated antigen isolated and structurally characterized from the human teratocarcinoma cell line 2102Ep (*24*) and subsequently identified in chicken pectoral muscle (*44*). This antigen contains a terminal trisaccharide epitope (NeuAcα2→3Galβ1→3GalNAc) defined by a monoclonal antibody directed to murine stage specific embryonic antigen 4 (SSEA4). In addition to forming the terminal epitope of SGG, this trisaccharide is also found on extended ganglio-series glycolipids, i.e., GM$_{1b}$, GD$_{1a}$, and GT$_{1b}$, as well as on sialosyl galactosyl-A (*45*) and the monosialylated derivative of the T-antigen (Thomsen-Friedenreich antigen) present on O-linked glycoproteins.

In our previous study, glycosphingolipids were extracted from vaginal epithelial cells collected from women who were nonsecretors and secretors of ABO blood group antigens (*12*). A radiolabeled *E. coli* isolate (R45) bound to two glycolipids present in the extract derived from nonsecretors but not from secretors when separated on TLC plates. These glycolipids comigrated with SGG and DSGG standards and the higher migrating glycolipid (comigrating with SGG) stained positive with a monoclonal antibody that binds SGG. The presence of SGG in tissues of nonsecretors is consistent with the idea that an α2→3sialyltransferase preferentially sialylates the precursor galactosyl globoside in the absence of the α1→2fucosyltransferase encoded by the *Se* gene.

The data presented in this report describe for the first time the purification and unambiguous structural characterization of SGG from human kidney and support the hypothesis that SGG is an important physiological receptor for uropathogenic *E. coli* expressing *pap*-encoded adhesins. In a parallel study, we demonstrated that this human kidney derived SGG binds to wild-type uropathogenic *E. coli* strain R45 in addition to cloned *pap* adhesin-expressing *E. coli* strains representing all three classes of known P fimbrial adhesins (*13*). More important, all strains of *E. coli* assayed bind to SGG with high avidity when compared to closely related globo-series glycosphingolipids.

Earlier studies by Karr et al. (*47*) showed that *pap-2*-encoded P-fimbria purified from a pyelonephritic strain of *E. coli* bound to cryostat sections of human kidney and to human erythrocytes expressing the Luke (LKE) antigen (defined by anti-LKE serum) and that binding to kidney sections could be inhibited by preincubation with a monoclonal antibody to SSEA4 (defined by MAb MC813−70). They concluded that the receptor for the *pap-2* encoded P-fimbria was LKE on human erythrocytes and SSEA4 on human kidney. Although it has been suggested that LKE and SSEA4 are identical antigens, the relationship is limited to a common determinant recognized by both anti-LKE serum and MAb MC813−70. Regardless of the fact that this determinant is found on SGG, the presence of this glycolipid in human kidney or any other normal human tissue has until now only been speculative. A recent study described the fine specificity of a monoclonal antibody directed to DSGG (*48*). This MAb is able to discriminate between a common branched tetrasaccharide epitope (NeuAcα2→3Galβ1→3{NeuAcα2→6GalNAc) found on DSGG, GD1α, and a common mucin-type epitope widely distributed on glycoproteins such as glycophorin A. The differential antibody-binding ability of this structure is thought to be dependent on its carrier glycoconjugate and branched characteristics. Monoclonal antibodies specific for globo-series ABH antigens (*49, 50*) as well as SGG are unavailable, and the difficulty in generating specific MAbs to these structures may be due to their linear characteristics. Unfortunately, until such MAbs are available, studies involving their tissue distribution will be extremely difficult. The results of this investigation clearly illustrate the presence of SGG in human kidney and may suggest a chemical basis for the increased risk of UTI in nonsecretors.

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HOST FACTORS IN SUSCEPTIBILITY TO URINARY TRACT INFECTIONS

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1. EPIDEMIOLOGY OF URINARY TRACT INFECTIONS IN WOMEN

Acute uncomplicated urinary tract infections (UTIs) occur in millions of young women each year and the majority of these infections are caused by Escherichia coli.

The frequency of this clinical problem is illustrated by the results of a recent large study (323 person-years of observation) of UTI among women followed in a university health clinic or enrolled in a health maintenance organization.[1] This study demonstrated an annual incidence of acute cystitis of 0.5 to 0.7 episodes per person-year.[1] In addition, from previous studies, it is estimated that 20–40% of women who experience an initial urinary tract infection (UTI) go on to develop recurrent infections.[2] Since bacteriuria is more frequent among older women,[3] recurrent UTI (RUTI) may be even more common among women over age 60. An estimated 10 to 15% of women in this age group have frequent recurrences.[4]

2. KNOWN FACTORS LEADING TO AN INCREASED SUSCEPTIBILITY TO UTI

Among selected populations of pre- and post-menopausal women, certain underlying factors predictably may lead to an increased susceptibility to UTI. For example, women of any age with a history of renal stones, urinary tract structural abnormalities, underlying medical conditions causing bladder dysfunction, or whose urinary tracts have been instrumented have an increased risk of recurrent infections. Certain functional and anatomic abnormalities of the urinary tract, such as post-void residual urine, reduced blad-

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der tone and/or prolapse, uterine prolapse, or the alterations in the normal vaginal flora towards a uropathogen-predominant state as a result of estrogen deficiency[5,6] are more common among post-menopausal woman and appear to increase susceptibility to RUTI.

3. RECURRENT UTI IN WOMEN WITH NORMAL URINARY TRACTS

Most otherwise healthy pre-menopausal women with RUTI do not have anatomic or functional abnormalities of the urinary tract. Thus, in nearly all such women, cystography and intravenous pyelography are normal.[7,8] In addition, among post-menopausal women, the above-mentioned structural and functional factors predisposing to recurrent infections are not universally present among and thus do not account for all recurrent infections among older women. Thus, it is not clear why recurrent UTI develops in some women whose urinary tracts appear to be normal from a functional and anatomical standpoint develop recurrent UTI, while other such women are spared.

4. COLONIZATION PRECEDING UTI

Colonization of the vaginal and periurethral mucosa with the infecting bacteria appears to be a necessary prerequisite to E. coli UTI.[9] Several lines of evidence suggest that susceptibility to colonization and other aspects of the interaction between the infecting uropathogenic E. coli and women's uroepithelial cells are key to understanding the increased susceptibility to RUTI among otherwise normal young women. Women with RUTI have prolonged vaginal colonization mucosa with uropathogenic E. coli, even between times of active infection.[10] When vaginal, buccal, and voided uroepithelial cells were collected from women with a history of recurrent UTI and tested in bacterial adherence assays, three-fold more E. coli adhered to these women's cells as compared with cells from control women without a history of RUTI.[11] Thus, the urogenital mucosa of women with RUTI appears to be more prone to bacterial adherence and persistence when examined either macroscopically as an organ or on a cellular level. Taken together, these data suggest that vaginal and uroepithelial cells from women with a history of recurrent UTI may express different or greater numbers of receptors for infecting E. coli as compared with cells from women without recurrences.

5. ADHESINS IN THE PATHOGENESIS OF E. COLI UTI

Adherence to the urothelial mucosa by E. coli adhesins such as pap family of adhesins, Type 1 fimbriae, and the Dr adhesin plays a key role in the pathogenesis of uncomplicated E. coli UTI.[12] In epidemiological studies, 50–65% of strains from women with cystitis possess pap adhesins vs. 10–15% of fecal isolates from patients without UTI.[12] Thus, pap adhesins are significantly associated with strains causing acute cystitis and are present in the majority of these strains. Variations in the DNA sequences of the adhesin define three classes of pap-related adhesins: class I, exemplified by the cloned isolates containing $papG_{J96}$; class II, represented by isolates expressing $papG_{IA2}$; and class III, such as isolates expressing $prsG_{J96}$.[12,13] The pap-encoded adhesins bind to specific glycosphingolipids (GSLs) in vitro;[14] our studies have focused on studying the expression of these compounds in urogenital tissues.

6. GLYCOSPHINGOLIPIDS AS UROEPITHELIAL CELL RECEPTORS FOR E. COLI

Glycosphingolipids (GSLs) are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane.[15] These molecules serve as antigens and cell surface markers of differentiation and oncogenesis.[15] GSLs are involved in mammalian cell-cell interactions and they serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including uropathogenic E. coli.[15]

Based on common carbohydrate core structures the GSLs are classified into families, such as the globoseries GSLs (Table 1).[16] This family of GSLs is characterized by a common internal galactose-α-1-4 galactose moiety which is the minimal receptor for uropathogenic E. coli expressing pap-encoded adhesins.[14] GSLs are synthesized by specific glycosyltransferases which sequentially link monosaccharides to specific carbohydrate core chains.[15] The expression of GSLs in a given tissue is determined by the specificities and quantities of these glycosyltransferases, the availability of substrate molecules for the enzymes, and competition among enzymes with the same substrate.[15] Some glycosyltransferases are tissue-specific and are regulated by the secretor gene (Se) or by polymorphic genes such as the ABO blood group genes.[16] As a result, GSLs on the cell surface are highly specific to a given host and play an important role both in determining tissue tropism for microbial pathogens and an individual host's susceptibility to specific infectious diseases.

7. HOST GENETIC FACTORS IN RECURRENT UTI

Several epidemiological studies have demonstrated that women with a history of recurrent UTI are three to four times more likely to be nonsecretors of ABH blood group antigens than are women without such a history.[17–19] The secretor gene, Se, encodes a glycosyltransferase which transfers a fucose residue to specific acceptor molecules, resulting in detectable blood group antigens in the secretions of people carrying the gene.[20] The Lewis genes act on the same acceptor molecules and if both Lewis and secretor genes are active in a given tissue, the Lewis blood type will reflect the Lewis antigens found in the tissues.[20] Separate fucosyltransferases are responsible for the synthesis of ABH blood group antigens on erythrocytes, as determined by the individual's ABO type.[20] Individuals who lack the secretor gene thus do not synthesize GSLs containing ABH blood groups epitopes in tissues under control of Se and instead may synthesize alternative epitopes.

Table 1. Structures of globoseries glycosphingolipids

GSL	Structure	Source
CTH	Gal-1-4 Galβ1-4 Glcβ1-1cer	human erythrocytes[21]
Globoside	GalNAcβ1-3 Gal-1-4 Galβ1-4 Glcβ1-1cer	human erythrocytes[21]
Gal globoside	Galβ1-3 GalNAcβ1-3 Gal-1-4 Galβ1-4 Glcβ1-1cer	human kidney[24]
SGG	NeuAc-2-3 Galβ1-3 GalNAcβ1-3 Gal-1-4 Galβ1-4 Glcβ1-1cer	human kidney[24]
DSGG	NeuAc-2-3 Galβ1-3 (NeuAc-2-6) GalNAcβ1-3 Gal-1-4 Galβ1-4 Glcβ1-1cer	human kidney[24]

8. GENETICALLY DETERMINED HOST CELL RECEPTORS FOR UROPATHOGENIC E. COLI

In our investigations, we integrated three areas of prior investigation by our group and others: (1) epidemiological observations regarding the association of the nonsecretor state with RUTI, (2) the propensity of women with a history of UTI to have increased degrees of vaginal E. coli colonization as compared with control subjects; and (3) laboratory observations about the adherence and bacterial binding characteristics of uropathogenic E. coli. At the onset of our investigations, it was unknown whether the secretor gene influenced the expression of and GSL in vaginal tissue nor if the synthesis of any globoseries GSL was influenced by this gene. To address these issues, we collected vaginal epithelial cells from secretors and nonsecretors and extracted the GSL fractions from vaginal epithelial cells pooled in equal quantities from women in the two groups.[21] We demonstrated that cells from nonsecretors express two unique globoseries GSLs, sialosyl galactosyl globoside (SGG) and disialosyl galactosyl globoside (DSGG).[21] These conclusions were drawn from a series of studies using several methods. First, we investigated the GSL fractions extracted from the secretors and nonsecretors, respectively, to identify GSL moieties that were capable of binding uropathogenic E. coli expressing pap-encoded adhesins.[21] We used high-performance thin layer chromatography (HPTLC) bacterial overlay assays, in which the GSLs were separated on HPTLC by size and then overlaid with a suspension of a metabolically labeled, wild-type E. coli R45[21] which expresses a Class II pap-encoded adhesin.[22] These studies showed two compounds reacting with bacteria that were found among GSLs extracted from nonsecretors' vaginal epithelial cells but not from GSLs from secretors' cells; the compounds migrated in positions corresponding with SGG and DSGG.[21] These bands were then purified from HPTLC plates and the identity of the compounds was confirmed using HPTLC immunostaining, radioimmunoassays and immunohistochemical staining using a monoclonal antibody directed against SGG.[21] These studies demonstrated for the first time that the secretor gene influences the biosynthesis of globo-series GSLs in the vaginal epithelium and suggested that genetically determined differences in receptor moieties in this tissue might explain the increased susceptibility of nonsecretors to UTI.[18,19,21,23]

In a more recent study, we demonstrated that SGG and DSGG are expressed in normal human kidney by isolating and purifying the compounds from this tissue.[24] This was accomplished through an exhaustive series of standard purification steps[25,26] to produce a neutral fraction and 0.05 M, 0.15 M, and 0.45 M ammoniun acetate fractions, the latter containing charged GSLs such gangliosides. Normal phase silica gel high-performance liquid chromatography (HPLC)[27] was then used to further separate the charged fractions. SGG and DSGG were identified and purified from these HPLC fractions by repeated cycles of HPTLC immunostaining[28,29] using specific MAbs[21,30] to identify the compounds, bacterial overlay assays[21] using uropathogenic E. coli carrying pap-encoded to identify globoseries GSLs, HPTLC in multiple solvent systems, and preparative HPTLC.[31] Figures 1A and 1B show examples of steps in this purification procedure, in which bacterial overlay assays using uropathogenic E. coli R45, expressing a pap-encoded adhesin, were performed to identify fractions containing relevant charged globoseries compounds, such as SGG and DSGG. When SGG and DSGG were purified, definitively determined the structure of purified SGG from human kidney using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), mass spectroscopy and linkage analysis.[32]

Next, we found that SGG and DSGG isolated from human kidneys bound each of 5 representative wild-type or recombinant uropathogenic E. coli expressing P fimbriae bearing pap-encoded adhesin Class I, II, or III, demonstrating that these are relevant bacterial

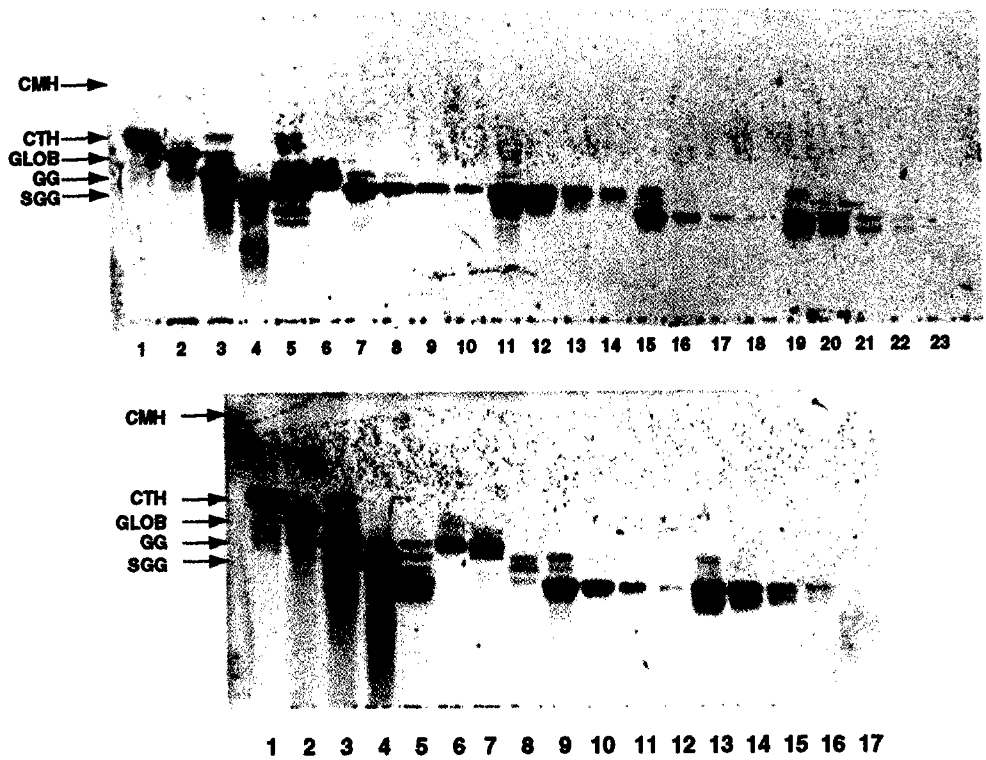

Figure 1. Purification procedures for isolating SGG and DSGG from human kidney. As described above, SGG and DSGG were purified from the upper phase GSL fractions through anion exchange chromatography followed by normal phase silica gel high-performance liquid chromatography (HPLC). To assess the relative amounts and purity of SGG or DSGG in specific HPLC fractions separated from the 0.15 M ammonium acetate and 0.45 M ammonium acetate fractions from human kidney, HPTLC bacterial overlay and immunostaining assays were used. Shown are autoradiographs from two examples of profiles of relevant HPTLC fractions using bacterial overlay assays with metabolically [^{35}S]methionine-labeled E. coli R45, which specifically binds globoseries GSLs. (A, top) 0.15 M ammonium acetate fractions from HPLC. Lane 1: ceramide trihexaosyl (CTH; Gb3) standard; Lane 2: globoside (GLOB; Gb4) standard; Lane 3: galactosyl globoside (GG) standard; Lane 4: sialosyl galactosyl globoside (SGG) standard; Lane 5: total 0.15 M ammonium acetate fraction (before HPLC); Lane 6: pooled 0.15 M ammonium acetate fractions 35–41; Lanes 7–10: pooled fractions 42–45, undiluted (Lane 7) and diluted 1:5, 1:25; and 1:125 (Lanes 8,9 and 10, respectively); Lanes 11–14: pooled fractions 46–51; undiluted (Lane 11) and diluted 1:5, 1:25; and 1: 125 (Lanes 12, 13 and 14, respectively); Lanes 15–18: pooled fractions 52–53; undiluted (Lane 15) and diluted 1:5, 1:25; and 1: 125 (Lanes 16,17 and 18, respectively); Lanes 19–22: pooled fractions 54–62; undiluted (Lane 19) and diluted 1:5, 1:25, and 1: 125 (Lanes 21 and 22, respectively); Lane 23: ceramide monohexosyl (CMH) standard. (B, bottom) 0.45 M ammonium acetate fraction from HPLC. Lane 1: ceramide trihexaosyl (CTH; Gb3) standard; Lane 2: globoside (GLOB; Gb4) standard; Lane 3: galactosyl globoside (GG) standard; Lane 4: sialosyl galactosyl globoside (SGG) standard; Lane 5: total 0.45 M ammonium acetate fraction (before HPLC); Lane 6: pooled fractions 20–42; Lane 7: pooled fractions 43–53; Lane 8: pooled fractions 54–56; Lanes 9–12: pooled fractions 57–58; undiluted (Lane 9) and diluted 1:5, 1:25, and 1:125 (Lanes 10, 11 and 12, respectively); Lanes 13–16: pooled fractions 59–64; undiluted (Lane 13) and diluted 1:5, 1:25, and 1:125 (Lanes 14, 15 and 16 , respectively); Lane 17: ceramide monohexosyl (CMH) standard.

binding entities for any of the known pap-encoded adhesins of E. coli.[24] To further investigate the potential relevance of the expression of these GSLs in the vaginal epithelium of nonsecretors, we metabolically radiolabeled the five representative E. coli isolates expressing pap-encoded adhesins and measured their relative binding affinities to serial dilutions of SGG and DSGG as well as to globotriaosylceramide (Gb_3) and globotetraosylceramide (Gb_4), two other globo-series GSLs present in urogenital tissues.[24] We found that each of the 5 representative E. coli isolates bound to SGG with the highest apparent avidity as compared with binding to DSGG, Gb_3 and Gb_4. These studies further suggest that SGG likely plays an important role in the pathogenesis of UTI and that its presence may account for the increased binding of E. coli to uroepithelial cells from nonsecretors and for their increased susceptibility to recurrent UTI. Further studies are needed to more extensively define the expression of SGG and DSGG in epithelial tissues throughout the urogenital tract. We are presently studying the GSL composition of normal human bladder epithelium, including investigating for the presence of SGG and DSGG.

9. ACQUIRED HOST FACTORS PREDISPOSING TO RUTI

Acquired factors may also affect susceptibility to UTI. The possibility that time-limited exposures or behaviors may predispose to RUTI is raised by observational studies of the natural history of UTI in young women have characterized by a temporal clustering phenomenon, in which several month periods marked by repeated infections are followed by infection free intervals.[33] Although it is not clear which specific factors might be responsible for such clusters of recurrent UTI, some of the same exposures and behaviors that increase the risk of sporadic episodes of acute uncomplicated UTI may be important. For example, multiple studies have shown that use of the diaphragm with spermicide for contraception[1,17,34–38] and recent sexual intercourse[1,17,35,38–41] greatly magnify the risk of sporadic UTI. The effect of spermicide use, with or without a diaphragm, appears to be mediated by increasing vaginal colonization with E. coli and other uropathogens,[17,36,37] perhaps through a selective microbicidal effect on the normal, protective vaginal flora such as lactobacilli.[36] Similarly, the antecedent use of antibiotics, either for treatment of a previous UTI or for other infections in the period 15 to 28 days before onset of UTI increases the proximal risk of for UTI by 2.57 to 5.83.[42] This exposure may also act by altering the indigenous urogenital flora towards a uropathogen-dominated flora.[10,43]

Many hygiene- or personal habit-related behaviors that might be postulated to affect the risk of UTI have not been thoroughly investigated or existing studies have produced conflicting results. Studies of women's voiding habits, fluid intake and the temporal sequence of micturition before and/or after intercourse are notable in this regard.[1,38,41,44,45] Behaviors such as perineal cleansing and the direction of wiping after bowel movements, menstrual products used, douching, and the use of vaginal deodorant products, have not been demonstrated to influence the risk of UTI.[38,39,41] Ongoing large epidemiological studies[46] will likely clarify whether personal habits and hygiene alter the risk of UTI.

10. SUMMARY

In summary, a variety of intrinsic and acquired factors influence the risk of RUTI in otherwise normal women, including history of prior UTIs, the woman's genetic background, and exposures to spermicides, sexual activity and antibiotics. Further studies are directed towards understanding the interplay between these factors and their relative im-

portance among various subpopulations of women with RUTI, such as otherwise healthy pre- and post-menopausal women.

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Prevention of recurrent urinary-tract infections in women

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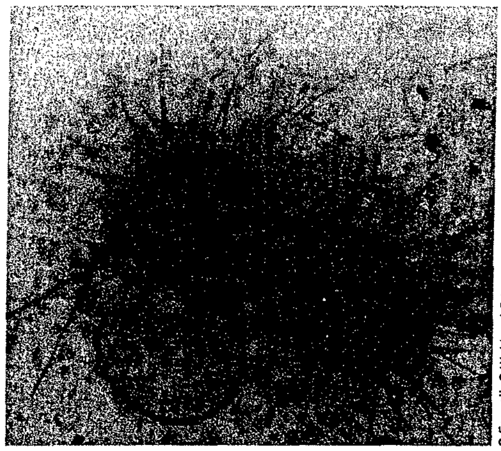
Prevention of recurrent urinary-tract infections in women

Acute cystitis is the commonest form of urinary-tract infection (UTI). Women are disproportionately affected, the annual incidence among young women in the USA being 0·5–0·7 episodes per person-year.[1] Furthermore, about 25% of women who have had an episode of acute cystitis develop frequent recurrent infections.[2] Since recurrent UTIs are a substantial burden to society in cost of diagnosis and treatment, time lost from work, and morbidity, research efforts aim to elucidate factors predisposing to recurrent UTI and to develop methods of preventing these infections. These efforts are especially important for women with recurrent UTI, because most of them have no demonstrable underlying abnormalities of the urinary tract. At each step in the pathogenesis of UTI, key host or bacterial factors influence susceptibility to UTI. A critical step is colonisation of the vaginal and periurethral mucosa with the infecting uropathogen, most commonly Escherichia coli. At the cellular level, bacterial factors that influence initial adherence between uropathogen and urogenital mucosa include the expression of pili such as P fimbriae, which are over-represented among isolates from patients with cystitis,[3] and type I fimbriae, which are present in all *E coli* and probably have a role in promoting initial colonisation.[4] Little difference has been found in virulence characteristics between *E coli* infecting women with sporadic UTI and those infecting women with recurrent infection.[5] However, the receptor repertoire for these organisms on host urogenital cells seems to influence susceptibility. Compared with women without recurrent infection, those with such infections have longer durations of vaginal colonisation with uropathogenic *E coli* and three-fold more *E coli* adhering to vaginal, buccal, and voided uroepithelial cells.[6]

In addition, women with recurrent UTI are three to four times more likely to be non-secretors of ABH blood-group antigens than are women without recurrent infections.[7] The secretor gene encodes one of the many glycosyltransferases that determine the carbohydrate composition of cell-surface glycoproteins and glycosphingolipids, some of which also serve as binding sites for uropathogenic *E coli*.[8] The vaginal epithelium of non-secretors, but not that of secretors, expresses two extended-chain glycosphingolipids that bind uropathogenic *E coli* more avidly than do other glycosphingolipids.[9,10] Thus, genetically determined differences in urogenital cell-receptor availability and binding characteristics may influence susceptibility to recurrent UTI.

Only certain features of the mucosal host response to bacterial attachment and infection have been investigated. Interleukins 6 and 8 are elaborated during UTI, presumably by the uroepithelium.[11] In women with acute cystitis, systemic immune responses are very weak, and local antibody responses are short-lived. Much more detailed knowledge of the role of the host immune response in sporadic and recurrent UTI is needed for the development of vaccines against these infections. Urogenital tissues contain antimicrobial peptides,[12] but their role in UTI is unknown.

The normal microbial ecology of the vagina may also be an important factor in the prevention of UTI. Several factors that disrupt the normal flora promote the



C Fennell, C Wobbe, J C Lara

Piliated uropathogenic *Escherichia coli*
Tube-like structure is the flagellum, which seems to have been detached from the bacterium.
Magnification x20 000, reduced by about 40%.

development of a uropathogen-predominant vaginal flora as well as a propensity to recurrent UTI. Such factors include the use of spermicides for contraception,[13] the recent use of β-lactam antimicrobials,[14] and the post-menopausal state unsupplemented by exogenous oestrogen.[15] Anatomical and behavioural factors in the patient also influence the likelihood of recurrent UTI, although some of these have been investigated only with respect to risk of sporadic UTI. Anomalies of urinary tract structure, urinary stones, as well as functional abnormalities associated with ageing, such as reduced bladder tone, post-void residual urine, and and bladder or uterine prolapse, may predispose to infection. Among behavioural risks, sexual intercourse is a major factor in predisposing women to the development of both sporadic and recurrent UTI, and this risk is magnified with the use of spermicide for contraception.[1] Whether micturition immediately after intercourse is protective is controversial.

Prophylactic use of antimicrobial agents is the cornerstone of prevention of recurrent UTI. Of the low-dose prophylactic regimens that have been used,[7] continuous low-dose prophylaxis, as described in a recent study by W Brumfitt and J M T Hamilton-Miller,[16] is the longest established. This strategy consists of daily or thrice-weekly subtherapeutic doses of an antimicrobial agent effective in the treatment of established UTI. Prophylactic doses of antimicrobials may also be given after intercourse.[7] Trimethoprim, trimethoprim-sulfamethoxazole, and norfloxacin have been tested the most and in the best studies.[7] Brumfitt and Hamilton-Miller used nitrofurantoin, which was one of the earliest agents to be used for prophylaxis; use of this drug in established infection is undergoing a revival. The design and results of their study showed some of the principles and problems associated with antimicrobial prophylaxis of UTI. Patients entering the study had a high frequency of recurrence, which was substantially reduced by prophylaxis. Characteristic of prophylactic regimens containing nitrofurantoin is the frequency of side-effects, including those necessitating the cessation of treatment. Serious side-effects, however, were rare. On the other hand, the faecal flora was not altered in terms of either the development of resistant organisms or loss of normal flora. Regimens including other antimicrobial agents,

such as β-lactams, may cause fewer side-effects but they drastically alter the faecal and vaginal flora.[7] Lastly, although Brumfitt and Hamilton-Miller report some "clinical improvement" for a while after the end of prophylaxis, in general, antimicrobial prophylaxis has no long-term effect on the natural history of recurrent UTI.[7]

What alternative strategies might be developed to enhance the effect of prophylactic antimicrobial therapy or to replace it? New preventive strategies resulting from studies into the molecular basis of host-parasite interactions and the role of the vaginal microbial ecology in UTI might include the development of soluble carbohydrate inhibitors for the inhibition of bacterial adhesion to the urogenital mucosa.[12,17] If disruption of the normal vaginal flora and loss of normally predominant vaginal lactobacilli is proven to be associated with recurrent UTI,[18] lactobacillus suppositories designed to promote this normal state will be investigated. One group is developing a vaccine against UTI, based on *E coli* type I fimbrial components.[19] Most of these measures are in the early stages of development. Modification of some behavioural risks, such as the use of spermicide, might, however, be effective in some cases.

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Clonal Relationships and Extended Virulence Genotypes among *Escherichia coli* Isolates from Women with a First or Recurrent Episode of Cystitis

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To identify bacterial predictors of recurrence and/or persistence in acute cystitis, extended virulence genotypes were compared with clonal background and epidemiologic status among 74 *Escherichia coli* urine isolates from women with first or recurrent episodes of urinary tract infection (UTI). Sequential isolates from patients with recurrent UTI were classified, using macrorestriction analysis, as having caused an isolated recurrence versus a single or multiple same-strain recurrences. *papA*, *papG* allele II, *iha*, and *iutA* predicted multiple same-strain recurrences, whereas *nfaE* and the absence of *sfaS* or *fyuA* predicted isolated recurrences. Phylogenetic group B2 accounted for 70% of isolates and for most of the putative virulence factors (VFs) studied. The meningitis-associated O18:K1:H7 clonal group comprised 18% of isolates, exhibited multiple VFs, and caused "once-only" recurrences less commonly than did other strains. These findings identify specific VFs and clonal groups against which preventive interventions might be beneficial and illustrate the importance of delineating pathogenetically relevant subgroups within the "recurrent cystitis" population.

Urinary tract infection (UTI) is a common health problem among adult women [1]. An estimated 25%–35% of women 20–40 years old have had ⩾1 episode of cystitis, the most common form of symptomatic UTI [2]. The enormous number of cases (~7 million annually in the United States [3]) results in a tremendous burden of clinic visits, direct health care costs (estimated as >1 billion dollars annually in the United States [4]), and lost productivity.

A substantial proportion of women who have an initial UTI episode experience frequent recurrences, the basis for which is understood incompletely [5, 6]. Certain behavioral or endogenous characteristics can predispose to UTI, including sexual intercourse (particularly with the use of spermicide-diaphragm contraception) [7, 8], prior antibiotic therapy [9], perineal anatomy [10], secretor status [11–13], P blood system phenotype [14], or a history of maternal UTI [15]. Whether the *Escherichia coli* strains that cause cystitis in women with a history of recurrent UTI possess traits (i.e., virulence factors [VFs]) that facilitate recurrence or persistence or, instead, are less virulent and rely on the host's underlying predisposition to UTI (e.g., a local immune defect) is unclear. In one study, among otherwise healthy women, certain characteristics of first-episode UTI isolates (i.e., the presence of *afa/dra* [afimbrial and fimbrial Dr-binding adhesins] and the absence of *cnf1* [cytotoxic necrotizing factor] and *sfa/foc* [S and F1C fimbriae]) predicted the occurrence of a second cystitis episode within 6 months [16]. In contrast, 2 other studies found that isolates from women with an established history of recurrent UTI differed minimally overall, compared with first-episode UTI isolates [17, 18].

Despite the selective use of pulsed-field gel electrophoresis (PFGE) in these studies to determine whether sequential isolates from a given patient represented the same strain or different strains, recurrent UTI isolates were not systematically categorized as having caused single episode, same-strain, or multiple same-strain recurrences [16–18]. We inferred that, since important virulence differences probably exist between these different epidemiologic subpopulations, it would be productive to stratify analyses of bacterial traits accordingly. Intuitively, the most virulent strains should be those that are able to cause multiple sequential same-strain UTI episodes. This presumably would reflect a special ability to persist in the host (including the vaginal and fecal flora) or in the host's immediate environment (such as other household members or fomites) and repeatedly re-invade the host. Strains of intermediate virulence

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should be those able to cause 2 recurrent UTI episodes (i.e., a single same-strain recurrence). The least virulent strains should be those able to cause only a single UTI episode, despite the host's seeming predisposition to UTI, as evidenced by the history of frequent recurrences. Strains that cause a first UTI episode in a host with no prior UTI history presumably should represent a mixture of these 3 categories; hence, in the aggregate, they would exhibit an intermediate level of virulence.

The availability of an epidemiologically well-characterized collection of 74 *E. coli* isolates from women with a first episode of UTI or recurrent UTI [17, 19] allowed us to test a series of specific hypotheses based on the above assumptions: specifically, we hypothesized that multiple same-strain recurrence isolates would exhibit a greater overall prevalence of VFs, and we inferred that any VFs (or phylogenetic groups) specifically associated with this cohort reflect high virulence. We hypothesized that the "single same-strain" recurrence group would exhibit an intermediate prevalence of VFs overall and would not be associated with specific VFs or phylogenetic groups. We hypothesized that "once-only" recurrence isolates would have a lower overall prevalence of VFs and that any VFs (or phylogenetic groups) specifically associated with this cohort reflect reduced virulence. Finally, we hypothesized that strains causing a first UTI episode in the aggregate would exhibit an intermediate prevalence of VFs and no VF-specific associations.

To test these hypotheses, we used newly developed broad-range virulence genotyping assays [20, 21] and amplification-based phylotyping methods [22, 23], to further characterize the 74 urine isolates [18]. Then we evaluated the distribution of bacterial traits among relevant epidemiologically and macrorestriction-derived subgroups within the population.

Methods

Strains. Seventy-four previously described *E. coli* urine isolates from women with a first or recurrent episode of cystitis were analyzed [17]. These strains have been characterized, with respect to PFGE type, mannose-resistant hemagglutination and hemolysin phenotypes, O:H serotype, and genotype for *papEFG* and the 3 *papG* alleles [17–19]. The *E. coli* reference (ECOR) collection [24] was obtained from Howard Ochman (University of Arizona, Tucson) and the American Type Culture Collection. Pyelonephritis isolate J96 (O4:K-:H5) [25] was provided by Barbara Minshew (retired; formerly University of Washington, Seattle). Pyelonephritis isolate 536 [26] was provided by Gabriele Blum-Oehler (Universität Würzburg, Würzburg, Germany). Calvin Kunin (Ohio State University, Columbus) provided O18:K1:H7 cystitis isolates [27, 28]. Neonatal meningitis isolates RS218 and 89-1449 (O18:K1:H7) [28] were provided by Kwang Sik Kim (Johns Hopkins University, Baltimore) and Lodewijk Spanjaard (Academic Medical Center, Amsterdam), respectively. Strains were stored in 20% glycerol at −70°C until use.

Virulence genotypes. Strains were tested for 27 different putative VF genes of extraintestinal pathogenic *E. coli* (ExPEC) [29], using dot-blot hybridization under stringent conditions, as described elsewhere [20, 22, 30]. Probes were generated and digoxigenin-labeled by using primers, as described elsewhere [20, 22, 30]. Adhesin genes included *papAH, papG, sfa/focDE, focG* (F1C fimbrial adhesin), *iha* (iron-regulated gene homologue adhesin) [30], *afa/draBC, bmaE* (M fimbriae), *gafD* (G fimbriae), and *nfaE* (nonfimbrial adhesin) [31]. Siderophores included *iutA* (aerobactin receptor), *fyuA* (yersiniabactin receptor), and *iroN* (a novel putative siderophore) [30]. Toxins included *hlyA* (hemolysin), *cnf1*, and *cdtB* (cytolethal distending toxin). Capsule genes included *kpsMT*-II and *kpsMT*-III (group II and III capsule synthesis). Miscellaneous VFs included *rfc* (O4 lipopolysaccharide synthesis), *cvaC* (colicin V), *traT* (serum resistance), *ibeA* (invasion of brain epithelium), and *malX*, a marker for a pathogenicity-associated island (PAI) from archetypal uropathogenic strain CFT073 [20, 32]. We tested for 4 VFs, including *kpsMT*-K1 (K1 capsule synthesis), *kpsMT*-II, *afa/draBC*, and *sfaS* (S fimbrial adhesin), by polymerase chain reaction (PCR) analysis, as described elsewhere [20]. Strains that were positive by probe but negative by PCR analysis for *kpsMT*-II were defined as K2 capsule–positive, as described elsewhere [20]. The aggregate VF score was the total number of VFs for which an isolate was positive, with proportional adjustment made for the multiple genes that were detected within the *pap* and *sfa/foc* operons.

Strains that were positive for any *pap* element were tested for 12 traditional alleles of *papA* plus a recently discovered *papA* variant (F48) by using a multiplex PCR-based assay, as described elsewhere [21]. All virulence genotypes were determined at least in duplicate, using boiled lysates prepared from 2 separate colonies of each strain. Discrepant results were investigated further, with additional determinations as needed.

Phylogenetic comparison with the ECOR collection. The 74 cystitis isolates were categorized as to phylogenetic group, on the basis of randomly amplified polymorphic DNA (RAPD) analysis [23, 33]. Genomic patterns were generated in duplicate for each strain, using 2 primers (1281, 5′-AACGCGCAAC-3′, and 1283 5′-GCGATCCCCA-3′) [23], to give a total of 4 genomic patterns per strain. Amplification conditions were as described by Berg et al. [33]. Patterns were combined in series to create a virtual composite pattern for each strain [22, 28, 34, 35]. Genomic patterns were generated in parallel for 10 control strains from the ECOR collection (i.e., 2 from each of the 5 major phylogenetic groups, as defined by Herzer, et al. [36], on the basis of allozymes at 38 loci). A similarity analysis was done on the basis of Pearson correlation coefficient values, as generated for comparisons of each cystitis isolate to each of 10 ECOR control strains [28, 34]. Any isolate with discrepant replicate determinations as to which ECOR strain it most closely matched underwent a third round of composite RAPD analysis. As an internal control, the ECOR strains were assessed for similarity to the other ECOR strains from the same phylogenetic group.

Serotypes. O:H serotypes were previously determined by the *E. coli* Reference Center (University Park, Pennsylvania) [18]. Thirteen isolates were defined as representing the O18:K1 clonal group. Of them, 11 were O18:K1, whereas 2 were O undetermined:K1 or O rough:K1, and, in the RAPD dendrogram, were placed within the cluster that contained predominantly O18:K1 strains. The six O6 isolates that were placed within the cluster that contained the 4 O6;F48 strains were defined as representing the O6;F48 clonal group.

Nicotinamide auxotrophy. Putative O18:K1 isolates were tested for growth at 30°C and 39°C on minimal media agar with and without supplementation with nicotinamide, as described elsewhere [27, 28, 37]. Strain J96 was used as a negative control, and strains RS218 and 89-1449 were used as positive controls for the outer membrane protein pattern (OMP) 6 and OMP 9 subclones, respectively, of *E. coli* O18:K1:H7. These strains exhibit distinctive nicotinamide auxotrophy phenotypes: that of the subclone OMP 6 is temperature independent (i.e., present at both temperatures), and that of the subclone OMP 9 is temperature dependent (i.e., present only at 39°C) [28, 37].

PFGE analysis. PFGE analysis was used previously to analyze 70 isolates recovered during prospective follow-up from the 24 patients with recurrent UTI [19]. These 70 isolates represented one acute UTI episode each for 3 patients (3 isolates total) and multiple UTI episodes (2–6 each) for 21 patients (67 isolates total). The 40 unique genotypes that were resolved by PFGE constituted the recurrent UTI strain population. Of these 40 genotypes, 3 were identified as having caused ⩾4 cystitis episodes in an individual patient; hence, they were defined as "multiple same-strain" recurrence isolates. These strains included R37 (O75:H7), R45 (O4:H5), and R55 (O118:H-undetermined). Fourteen of the genotypes were recovered only twice from a given patient; hence, they were defined as "single same-strain" recurrence isolates. The remaining 23 genotypes from patients with recurrent UTI were recovered only once each; hence, they were defined as "once-only" recurrence isolates.

Statistical methods. Comparisons of proportions between groups were tested by using Fisher's exact test, and comparisons of proportions within the same population were tested by using McNemar's test [38]. Correlations between traits were assessed by using Cramer's Φ, and comparisons between groups with respect to aggregate VF scores were tested by using the Mann-Whitney U test (2-tailed). Stepwise multivariate logistic regression analysis was used to identify VFs that were independently predictive of specific epidemiologic subgroups. The threshold for statistical significance was $P < .01$, with $P < .05$ considered to reflect borderline statistical significance.

Results

Epidemiologic distribution of VFs. In the population as a whole, the various VFs ranged in prevalence from 1% (*cdtB* and the K2 *kpsMT* II variant) to 94% (*fyuA;* table 1), with aggregate VF scores of 0–12 (median, 5.5). With respect to individual VFs, the multiple same-strain recurrence subgroup exhibited the highest prevalence for more VFs than did any other subgroup (13 vs. 3–9 VFs; $P < .05$, McNemar's test, for multiple same-strain vs. once-only recurrence isolates). Conversely, the once-only recurrent UTI subgroup exhibited the lowest prevalence for more individual VFs than did any other subgroup (13 vs. 1–7 VFs; $P < .01$, McNemar's test, for once-only vs. either single same-strain recurrence or first-episode isolates). Aggregate VF scores were highest among the multiple same-strain recurrence isolates (median, 10), were intermediate among the 14 single same-strain recurrence and the 34 first-episode isolates (median, 7.5 and 5.5, respectively), and were lowest among the 23 once-only recurrence isolates (median, 4).

For individual VFs, the 3 multiple same-strain recurrence isolates (compared with all other isolates) exhibited a significantly higher prevalence of *papA, papG* allele II, *iha,* and *iutA,* whereas the 23 once-only recurrence isolates exhibited a significantly lower prevalence of *sfaS* and *fyuA* and a significantly higher prevalence of *nfaE* (table 1). In contrast, the remaining 2 subgroups either did not differ significantly from other strains with respect to any VF (single same-strain recurrence subgroup) or differed weakly only with respect to *focG* and *fyuA* (first-episode subgroup; table 1).

Associations between VFs. Because various VFs were highly correlated with one another in an overlapping fashion (figure 1), stepwise multivariate logistic regression analysis was used to assess the independent associations of individual VFs with the extreme high- and low-virulence epidemiologic subgroups. This confirmed the presence of *papG* allele II, *iha,* and *papA* as independent predictors of multiple same-strain recurrence status and confirmed both the absence of *sfaS* and the presence of *nfaE* as independent predictors of once-only recurrence status (data not shown).

Phylogenetic relationships. According to composite RAPD analysis, the population consisted of 2 major phylogenetic clusters (clusters 1 and 2) comprising 52 (70%) and 22 (30%), respectively, of the isolates (figure 2). The distribution of the ECOR control strains revealed that cluster 1 corresponded mainly with ECOR phylogenetic group B2, whereas cluster 2 corresponded mainly with ECOR phylogenetic groups A, B1, and nonaligned strains. The group D ECOR strains were divided between clusters 1 (ECOR 39) and 2 (ECOR 48; figure 2).

The first-episode and the (aggregate) recurrent UTI isolates did not differ appreciably with respect to their distribution between cluster 1 (which included 68% and 73%, respectively, of each group) and cluster 2. All 3 multiple same-strain recurrence isolates were from cluster 1, however, and the 17 same-strain recurrence isolates were somewhat more likely to be from cluster 1 (14 [82%] of 17) than were the once-only recurrent UTI isolates (15 [65%] of 23) or all other isolates combined (38 [67%] of 57).

Phylogenetic distribution of VFs. Clusters 1 and 2 differed dramatically with respect to the prevalence of most of the VFs assessed (table 2). Each VF that exhibited a significant prevalence difference between clusters was more prevalent in cluster 1 than in cluster 2. VFs that exhibited the opposite trend (i.e., those somewhat more prevalent within cluster 2) included *nfaE* and *cvaC,* whereas *iutA* and *gafD* were similarly prevalent in the 2 clusters (table 2). Aggregate VF scores were much greater within cluster 1 than cluster 2 (median, 8.5 vs. 2; $P < .001$).

Within cluster 1, 2 prominent subclusters were evident at about the 80% similarity level (figure 2). One of these clusters (clone A) comprised 6 O6 isolates (8% of the total). The other cluster (clone B) comprised 13 putative O18:K1 isolates (18%

Table 1. Distribution of virulence factors (VFs), according to epidemiological category among 74 cystitis isolates of *Escherichia coli*.

		No. (%) of recurrent UTI, by subgroup[a]				
Associated trait	No. (%) with VF	Multiple same-strain recurrences[b] (n = 3)	Single same-strain recurrence[c] (n = 14)	Once-only recurrence[d] (n = 23)	Total recurrent UTI[e] (n = 40)	First-episode UTI[e] (n = 34)
papA	26 (35)	3 (100)[b]	7 (50)	5 (22)	15 (38)	11 (32)
F10 allele	16 (22)	2 (67)	3 (21)	2 (9)	7 (18)	9 (26)
F48 allele	5 (7)	1 (33)	0	3 (13)	4 (10)	1 (3)
papG	27 (36)	2 (67)	7 (50)	5 (22)	14 (35)	13 (38)
Allele II	4 (5)	2 (67)[b]	1 (7)	0	3 (8)	1 (3)
Allele III	23 (31)	0	6 (43)	5 (22)	11 (28)	12 (35)
sfa/focDE	27 (36)	1 (33)	7 (50)	5 (22)	13 (33)	14 (41)
sfaS	23 (31)	1 (33)	5 (36)	3 (13)[d]	9 (23)	14 (41)
focG	6 (8)	0	3 (21)	3 (13)	6 (15)[e]	0[e]
afa/draBC	3 (4)	0	1 (7)	0	1 (3)	2 (6)
iha	12 (16)	3 (100)[b]	2 (14)	2 (9)	7 (18)	5 (15)
bmaE	4 (5)	0	1 (7)	1 (4)	2 (5)	2 (6)
gafD	8 (11)	0	1 (7)	3 (13)	4 (10)	4 (12)
nfaE	10 (14)	0	1 (7)	7 (30)[d]	8 (20)	2 (6)
hlyA	27 (36)	2 (67)	7 (50)	5 (22)	14 (35)	13 (38)
cnf1	25 (34)	1 (33)	7 (50)	5 (22)	13 (33)	12 (35)
cdtB	1 (1)	0	0	0	0	1 (3)
iroN	32 (43)	2 (67)	6 (43)	7 (30)	15 (38)	17 (50)
fyuA	61 (82)	3 (100)	11 (79)	15 (65)[d]	29 (73)[e]	32 (94)[e]
iutA	21 (28)	3 (100)[b]	2 (14)	6 (26)	11 (28)	10 (29)
*kpsMT*II	46 (62)	3 (100)	9 (64)	11 (48)	23 (58)	23 (68)
K1	25 (34)	1 (33)	6 (43)	5 (22)	12 (30)	13 (38)
"K2"	1 (1)	0	0	0	0	1 (3)
*kpsMT*III	3 (4)	0	1 (7)	2 (9)	3 (8)	0
rfc	3 (4)	1 (33)	1 (7)	1 (4)	3 (8)	0
ibeA	18 (24)	0	6 (43)	4 (17)	10 (25)	8 (24)
cvaC	9 (12)	0	2 (14)	3 (13)	5 (13)	4 (12)
traT	45 (61)	1 (33)	7 (50)	14 (61)	22 (55)	23 (68)
malX	47 (64)	3 (100)	10 (71)	12 (52)	25 (63)	22 (65)

NOTE. UTI, urinary tract infection.

[a] As defined on the basis of pulsed-field gel electrophoresis analysis of sequential urine isolates from women in the recurrent UTI cohort.

[b] For multiple same-strain recurrence subgroup vs. all other isolates, $P = .04$ (*papA*), $P = .007$ (*papG* allele II), $P = .003$ (*iha*), and $P = .02$ (*iutA*). For other comparisons, $P > .05$.

[c] For single same-strain recurrence subgroup vs. all other isolates, $P > .05$ for all comparisons.

[d] For once-only recurrence subgroup vs. all other isolates, $P = .03$ (*sfaS*), $P = .008$ (*nfaE*), and $P < .02$ (*fyuA*). For other comparisons, $P > .05$.

[e] For first-episode UTI vs. all other isolates (i.e., total recurrent UTI population), $P = .03$ (*focG* and *fyuA*). For other comparisons, $P > .05$.

of the total). Each of these clonal groups exhibited a significantly higher prevalence of numerous individual VFs than did all remaining isolates ("all others" in table 3) or the remaining isolates from cluster 1 ("others, group 1 only" in table 3). Only *iutA* was significantly more prevalent among other strains than within clonal groups A and B (table 3). Aggregate VF scores were higher among members of clonal groups A and B (median, 11 and 12, respectively) than among all other isolates (median, 4; $P < .001$ vs. either clone A or B) or among other cluster 1 isolates (median, 6; $P = .003$ vs. clone A, and $P < .001$ vs. clone B). Clonal groups A and B exhibited a consensus virulence genotype that included the presence of a complete *pap* operon, *papG* allele III, *sfa/foc, sfaS, hlyA, cnf1, iroN, kpsMT* II, *traT*, and the PAI marker *malX* and the absence of *iutA*. They differed with respect to the F48 *papA* allele (specific to clone A) and the F10 *papA* allele, the K1 *kpsMT* II variant, and *ibeA* (all specific to clone B; table 3).

Although clonal group B consisted predominantly of first-episode UTI isolates (9 [69%] of 13), those of its representatives that did cause recurrent UTI were more likely to have caused same-strain recurrences (3 [75%] of 4) than were non–clonal group B recurrent UTI isolates (14 [39%] of 36). Thus, clonal group B isolates were significantly less likely than were other strains to have caused a (low virulence) once-only recurrence (1 [8%] of 13, vs. 22 [36%] of 61; $P = .05$).

Discussion

In the present study we defined extended virulence genotypes and phylogenetic relationships among urine isolates from

	Adhesins								Toxins		Siderophores			Capsules: *kpsMT*			Miscellaneous				
	pap				*sfa/*																
Adhesins	*A*	*G*	II	III	*focDE*	*sfaS*	*focG*	*nfaE*	*hlyA*	*cnf1*	*iroN*	*fyuA*	*iutA*	II	K1	K2	*rfc*	*cvaC*	*traT*	*ibeA*	*malX*
papA	n/a	++	+	++	++	++	-	-	++	++	++	+	-	++	++	-	-	-	+	++	++
F10	++	++	-	++	++	++	-	-	++	++	++	-	-	++	++	-	-	-	-	++	++
F48	+	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
papG			+	++	++	++	-	-	++	++	++	+	-	++	++	-	-	-	++	++	++
allele II				-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-
allele III					++	++	-	-	++	++	++	+	(++)	++	++	-	-	-	+	++	++
sfa/focDE						++	++	-	++	++	++	+	-	++	+	-	-	-	+	++	++
sfaS							-	-	++	++	++	+	-	++	+	-	-	-	++	++	++
focG								-	-	+	+	-	-	-	-	-	-	-	-	-	-
iha								-	-	-	-	-	++	-	-	-	-	-	-	-	-
gafD								++	-	-	-	-	-	-	-	-	-	-	-	-	-
Toxins																					
hlyA										++	++	+	-	++	++	-	-	-	+	++	++
cnf1											++	+	(++)	++	++	-	-	-	-	++	++
Siderophores																					
iroN												+	-	+	-	-	-	+	+	++	++
fyuA													-	++	++	-	-	-	-	-	++
iutA														-	-	-	-	-	-	-	-
Capsules																					
kpsMT II															-	-	-	-	+	++	++
kpsMT III															-	-	++	-	-	-	-
K1																-	-	-	++	++	+
Miscellaneous																					
rfc																		-	-	-	-
cvaC																			-	-	-
traT																				-	+
ibeA																					+

Figure 1. Pairwise correlations between virulence factors (VFs). Only those VFs that exhibited ⩾1 correlation with another VF at the $P<.01$ level are shown. Significance levels: −, $P>.01$; +, $P\leqslant.01$; and ++, $P\leqslant.001$. Parentheses indicate negative associations. *cnf1,* Cytotoxic necrotizing factor; *cvaC,* colicin V; F10 and F48, 2 *papA* (structural subunit) alleles; *focG,* F1C adhesin; *fyuA,* yersiniabactin receptor; *gafD,* G fimbriae; *hlyA,* hemolysin; *ibeA,* invasion of brain endothelium; *iha,* novel putative siderophore-adhesin; *iroN,* putative siderophore; *iutA,* aerobactin receptor; *kpsMT,* capsule synthesis (group II, group III, K1 variant, or K2 variant); *malX,* pathogenicity-associated island marker; *nfaE,* nonfimbrial adhesin; *pap,* P fimbriae; *papG,* P adhesin gene (including alleles II and III); *rfc,* O4 lipopolysaccharide synthesis; *sfa/focDE,* S and F1C fimbriae; *sfaS,* S fimbrial adhesin; *traT,* serum-resistance–associated gene.

women with a first episode of cystitis or recurrent cystitis. We found that differences between clinical groups were more numerous and more extreme when the recurrent UTI isolates were stratified by PFGE analysis according to whether they had caused any or multiple same-strain recurrent UTI episodes (as opposed to when they were analyzed collectively as a homogenous population).

Our findings illustrate the importance in epidemiologic studies of stratifying recurrent UTI isolates according to their same-strain or non–same-strain status [19, 39, 40]. The observed distribution of VFs mainly conformed to our inferred virulence strata, for which future experimental validation would be desirable. VFs were most prevalent among the multiple same-strain recurrence isolates, were least prevalent among the once-only recurrence isolates, and were of intermediate prevalence among the single same-strain recurrence isolates and the first-episode isolates (which presumably represent a mixture of the previous 3 groups).

VFs specifically associated with multiple same-strain recurrences (i.e., putative high-virulence VFs) included *papA, papG* allele II, *iha,* and *iutA.* In contrast, the association of *nfaE* with once-only recurrences implicated this adhesin as a putative low-virulence VF, whereas the negative associations of *sfaS* and *fyuA* with this subgroup identified these VFs as also representing high-virulence VFs. It may be that the high-virulence VFs identified in the present study, which are adhesins and siderophores, would be good targets for interventions designed to prevent either first episodes of UTI or multiple same-strain recurrences [8, 41–43]. In addition, typing of isolates from women with recurrent UTI for these genes might allow for some prediction as to whether multiple same-strain recurrences can be expected and whether host factors (e.g., defective local immunity or increased receptivity to bacterial attachment) versus bacterial factors are more important contributors to recurrence. This, in turn, might guide decisions regarding intensity or duration of antimicrobial therapy [39, 44], attention to potentially

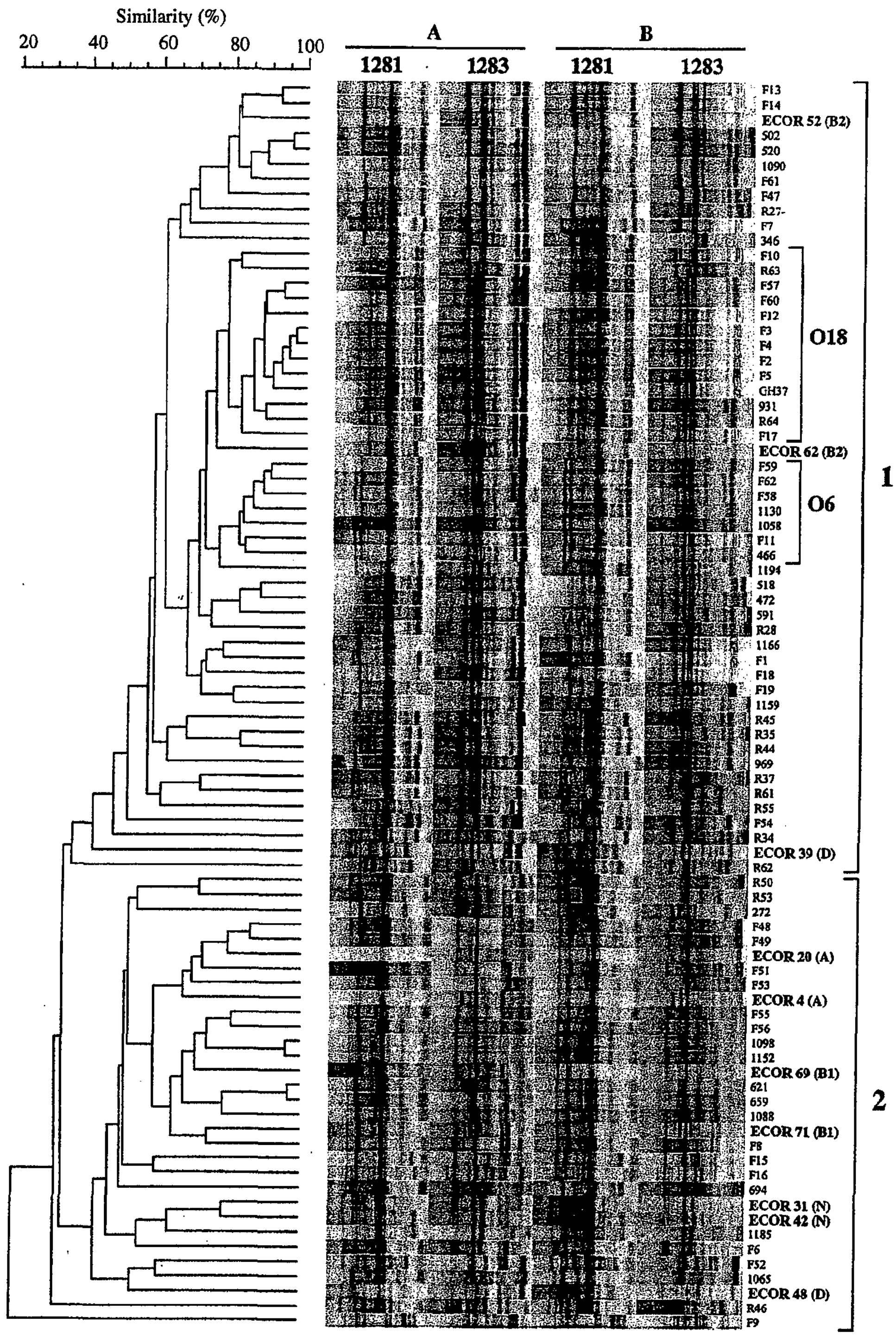

Figure 2. Random amplified polymorphic DNA (RAPD) dendrogram of 74 cystitis isolates and 10 *Escherichia coli* reference (ECOR) strains. Dendrogram is based on similarity relationships, according to composite genomic patterns from 2 rounds (A and B) of amplification with RAPD primers 1281 and 1283, with cluster analysis according to the unweighted pair group method with averaging. Gel tracks are computer reconstructions and, hence, underestimate the resolution of the original gel images. Major clusters 1 and 2 correspond with ECOR groups B2 vs. groups A, B1, and nonaligned strains, respectively. Group D strains are divided between the 2 clusters. Within cluster 1, brackets labeled "O6" and "O18" demarcate clonal groups A and B, respectively. ECOR strains are identified parenthetically as to phylogenetic group of origin.

reversible host factors [7, 13], genomic typing of serial isolates [19], or investigation for persisting internal or external reservoirs of such strains [19, 45, 46].

In a previous epidemiologic study that analyzed 9 VFs as predictors of recurrent UTI within 6 months of an initial UTI episode in otherwise healthy women, the only significant predictor was *afa/dra* (relative risk [RR], 2.3) [16]. In contrast, 3 VFs were associated significantly with absence of recurrence, including *cnf* (RR, 0.0), *hly* (RR, 0.1), and *sfa/foc* (RR, 0.25) [16]. All 4 recurrence episodes associated with *afa/dra* were same-strain recurrences. In that study, *afa/dra* was moderately prevalent in the population (14%), *sfa/foc* positivity was not further stratified as to *sfa* versus *foc,* and all patients were enrolled at the time of their first UTI episode [16]. In contrast, in the present study, *afa/dra* was uncommon (4%) and was not associated with recurrent UTI (whether same-strain or not); *sfaS* and *focG,* which were separately detected, exhibited opposing epidemiologic associations; single and multiple same-strain recurrences were differentiated from once-only recurrences; and isolates from patients with a history of multiple previous recurrences constituted 55% of the study population. Whether differences in study design, laboratory methods, and/or geographic locale account for the discrepant findings of these 2 studies is unknown.

Of note, NfaE-I shares 96% peptide homology with Dr-II, which, like other members of the AFA/Dra adhesin family, mediates adherence to tubular basement membranes and Bowman's capsule and promotes bacterial internalization into HeLa cells [47]. Internalization into host cells might be expected to confer same-strain recurrence capability [48], which would be consistent with the association of *afa/dra* with same-strain recurrence, as noted by Foxman et al. [16]. However, in the present study, *nfaE* actually was negatively associated with same-strain recurrence. This suggests that, despite their peptide homology, the NfaE-I and Dr-II adhesins may exhibit important functional differences and/or that the relationship between adhesin-mediated bacterial internalization into host cells and same-strain recurrent UTI is not straightforward. Also of note is the demonstrated in vitro ability of Dr adhesins to mediate bacterial adherence to neutrophils without triggering phagocytosis or killing [49]. Whether less favorable interactions with neutrophils could contribute to the seeming "anti-VF" associations observed with *nfaE* in the present study warrants investigation.

Another relevant previous study involved urine isolates from children treated for acute cystitis [39]. In that population, although *papG* allele II was the most prevalent *papG* allele and was associated with recurrence per se, *papG* allele III was significantly associated specifically with same-strain recurrence [39]. In contrast, in the present study, *papG* allele III was the most prevalent *papG* allele, and *papG* allele II was significantly associated with multiple same-strain recurrences. Differences between these 2 studies with respect to study design (treatment trial vs. observational study), host population (children with acute cystitis vs. women with first or recurrent episodes of UTI), and geographic locale (Cleveland vs. Seattle) provide several possible explanations for their discrepant findings with respect to which VFs predict recurrent UTI and, specifically, same-strain recurrence in patients with cystitis.

A noteworthy finding of the present study was that 2 large, VF-rich clonal groups accounted for 26% of the study population, a relatively large proportion, considering the tremendous diversity of *E. coli* clones [50–52]. The clonal group characterized by the O6 antigen and the F48 *papA* allele (clonal group A), which accounted for 8% of the population, also is prominent among canine UTI isolates [22, 53], in canine feces [54], and among human bacteremia isolates [20] (J. R. Johnson, J. Maslow, and T. T. O'Bryan, unpublished data). It corresponds with clonal group 2 of Cherifi et al. [55], which comprised extraintestinal isolates from humans and diverse animals, and includes archetypal pyelonephritis isolate 536 (O6:K15:H31) [26, 53], as confirmed for isolates from the present study by direct RAPD analysis in comparison with strain 536 (data not shown).

The O18:K1 clonal group (clonal group B), which accounted for 18% of the present study population, exhibited enhanced virulence in that its members were more likely than other isolates

Table 2. Distribution of virulence factors (VFs) in relation to phylogenetic group among 74 cystitis isolates of *Escherichia coli.*

		Prevalence of associated trait, by phylogenetic group, no. (%)		
Associated trait	No. (%) with VFs	Cluster 1 (n = 52)	Cluster 2 (n = 22)	P[a]
papA	26 (35)	26 (50)	0	<.001
F10 allele	16 (22)	16 (31)	0	.002
F48 allele	5 (7)	5 (10)	0	
papG	27 (36)	27 (52)	0	<.001
Allele II	4 (5)	4 (8)	0	
Allele III	23 (31)	23 (44)	0	<.001
sfa/focDE	27 (36)	27 (52)	0	<.001
sfaS	23 (31)	23 (44)	0	<.001
focG	6 (8)	6 (12)	0	
afa/draBC	3 (4)	3 (6)	0	
iha	12 (16)	11 (21)	1 (5)	
bmaE	4 (5)	4 (8)	0	
gafD	8 (11)	5 (10)	3 (14)	
nfaE	10 (14)	5 (10)	5 (23)	
hlyA	27 (36)	27 (52)	0	<.001
cnf1	25 (34)	25 (48)	0	<.001
cdtB	1 (1)	1 (2)	0	
iroN	32 (43)	28 (54)	4 (18)	.02
fyuA	61 (82)	49 (94)	12 (55)	<.001
iutA	21 (28)	15 (29)	6 (27)	
*kpsMT*II	46 (62)	45 (87)	1 (5)	<.001
K1	25 (34)	24 (46)	1 (5)	<.001
"K2"	1 (1)	1 (2)	0	
*kpsMT*III	3 (4)	3 (6)	0	
rfc	3 (4)	3 (6)	0	
ibeA	18 (24)	18 (35)	0	.001
cvaC	9 (12)	5 (10)	4 (18)	
traT	45 (61)	36 (69)	9 (41)	.04
malX	47 (64)	47 (90)	0	<.001
Cluster 1	52 (70)	NA	NA	

NOTE. NA, not applicable (comparison with self).

[a] For comparison of cluster 1 vs. cluster 2.

Table 3. Clonal distribution of virulence factors among 74 *Escherichia coli* isolates from women with acute cystitis.

Associated trait	Prevalence of associated trait, no. (%)				P[a]			
	Clone A (n = 6)	Clone B (n = 13)	All others (n = 55)	Others, group 1 only (n = 33)	Clone A, vs. all others	Clone B, vs. all others	Clone A, vs. others (group 1 only)	Clone B, vs. others (group 1 only)
papA	4 (67)	13 (100)	9 (16)	9 (27)	.02	<.001		<.001
F10 allele	1 (17)[b]	13 (100)[b]	1 (2)	2 (6)		<.001		<.001
F48 allele	4 (67)[c]	0[c]	1 (2)	1 (3)	<.001		.001	
papG	6 (100)	13 (100)	8 (15)	8 (24)	<.001	<.001	.001	<.001
Allele II	0	0	4 (7)	4 (12)				
Allele III	6 (100)	13 (100)	4 (7)	4 (12)	<.001	<.001	<.001	<.001
sfa/focDE	6 (100)	13 (100)	8 (15)	8 (24)	<.001	<.001	.001	<.001
sfaS	4 (67)	12 (92)	7 (13)	8 (24)	.008	<.001	.001	<.001
hlyA	6 (100)	13 (100)	8 (15)	8 (24)	<.001	<.001	.001	<.001
cnf1	6 (100)	13 (100)	6 (11)	6 (18)	<.001	<.001	<.001	<.001
iroN	6 (100)	12 (92)	14 (25)	9 (27)	.001	<.001	.002	<.001
iutA	0	0	21 (38)	15 (45)		.006		.004
kpsMT II	6 (100)	13 (100)	27 (49)	26 (79)	.03	<.001		
K1	1 (17)[b]	13 (100)[b]	11 (20)	10 (30)		<.001		<.001
ibeA	0[b]	11 (85)[b]	7 (13)	7 (21)		<.001		<.001
traT	5 (83)	13 (100)	27 (49)	18 (55)		<.001		.004
malX	6 (100)	13 (100)	28 (51)	28 (85)		.03		.001

NOTE. "Others" refers to strains not in clone A or clone B.
[a] P values shown only if <.05. Only traits yielding $P < .05$ for ≥1 comparison are included in the table.
[b] For clone A vs. clone B, $P = .001$.
[c] For clone A vs. clone B, $P = .004$.

to have caused first episodes of UTI or a same-strain recurrence and were less likely to have caused once-only recurrence. By serotype and VF profile, these strains appeared to be highly similar to the O18:K1:H7 strains that Kunin et al. [27] isolated from the urine of 15% of women with cystitis in Columbus, Ohio, and to archetypal neonatal meningitis isolates RS218 and C5 [28, 56]. The suspected similarities were confirmed directly by RAPD analysis, which showed selected representatives of clonal group B to be indistinguishable from representatives of these comparison *E. coli* O18:K1:H7 populations (data not shown). In addition, all the members of clone B (O18:K1) exhibited nicotinamide auxotrophy at both 30°C and 39°C, which is consistent with membership in the OMP 6 rather than the OMP 9 subclone of *E. coli* O18:K1:H7 [28, 37]. Together with previous data regarding the Kunin cystitis isolates [28], these findings suggest that the OMP 6 subclone of *E. coli* O18:K1:H7, which accounts for almost all O18:K1 neonatal meningitis isolates in the United States [37, 57], also is a major contributor to cystitis in North American women.

Our findings of extensive associations among VFs are consistent with previous observations regarding many of the specific correlations identified here [20, 30, 58], which thus appear to represent general trends among ExPEC. As noted elsewhere [30], some traits that are known to be genetically linked in an index strain (such as *iha* and the PAI marker *malX*, which occur within 2 kb of one another on a PAI in archetypal strain CFT073 [59]) may not be statistically associated with one another at the population level and may even exhibit divergent associations with other VFs [30]. This provides additional evidence that PAIs undergo remodeling and that linkages between VFs are not fixed [30, 58].

We used a novel approach to assess linkages between VFs by analyzing the total number of different VFs associated with each individual VF. We found that, unlike other VFs, *nfaE* [31], which appeared epidemiologically to be a low-virulence VF because of its positive association with the once-only recurrence subgroup, was associated with a slight decrease in aggregate VF scores (data not shown), which might contribute to its seemingly low-virulence behavior.

In contrast, although several of the putative high-virulence VFs were associated with significantly increased aggregate VF scores, these effects were not large, compared with the effects observed with other VFs that were not epidemiologically associated with virulence. Indeed, for 2 of the putative high-virulence VFs *(iha* and *iutA),* VF scores were not affected at all by the presence or absence of the trait (data not shown). Thus, although some of the enhanced virulence associated with *sfaS, papA, papG* allele II, and *fyuA* might be attributable to these VFs serving as markers for a more robust VF repertoire in general, with *iha* and *iutA* in particular, enhanced virulence could not be ascribed simply to an increased number of VFs. This strengthens the case for *iha* and *iutA* themselves and, from the multivariate analysis, for *iha* in particular, as direct agents of enhanced virulence. Confirmation of these findings in a larger population of multiple same-strain recurrence isolates and experimental validation of these epidemiologically derived predictions are needed.

In summary, we found through a combined molecular and phylogenetic analysis of *E. coli* isolates from women with first-episode and recurrent UTI that certain putative VFs *(papA, papG* allele II, *iha, iutA, sfaS,* and *fyuA)* were epidemiologically associated with enhanced virulence, whereas *nfaE* was associ-

ated with reduced virulence. Stratification of recurrent UTI isolates according to multiple same-strain recurrences, single same-strain recurrence, or isolated recurrence episode revealed potentially important virulence profile differences that otherwise were not apparent. *E. coli* phylogenetic group B2 and, in particular, its 2 constituent clonal groups that correspond with archetypal ExPEC strains 536 (pyelonephritis: O6:K53:H31) and RS218 (neonatal meningitis: O18:K1:H7) predominated in the study population and exhibited a markedly higher prevalence of most VFs than did other strains. The O18:K1 clonal group, in particular, exhibited enhanced virulence behavior. The extensive associations documented between VFs underscored the importance of cautious analysis and interpretation of molecular epidemiologic data and the need for experimental assessment of the contribution of specific putative VFs to the pathogenesis of extraintestinal *E. coli* infections.

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